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PhD Thesis

Antioxidant Minerals in Alternative Diets for Gilthead Sea Bream (*Sparus aurata*):

Biomarkers, Sources and Adequate Levels

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Doctorado en Acuicultura Sostenible y Ecosistemas Marinos

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INFORMA,

Que la Comisión Académica del Programa de Doctorado, en su sesión de fecha ..... tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "**ANTIOXIDANT MINERALS IN ALTERNATIVE DIETS FOR GILTHEAD SEA BREAM (*Sparus aurata*): BIOMARKERS, SOURCES AND ADEQUATE LEVELS**" presentada por el doctorando **D. DAVID DOMÍNGUEZ MONTESDEOCA** y dirigida por las Doctoras **MARÍA SOLEDAD IZQUIERDO LÓPEZ** y **LIDIA E. ROBAINA ROBAINA.**

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento de Estudios de Doctorado (BOULPGC 7/10/2016) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a...de.....de dos mil.....

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Título de la Tesis

ANTIOXIDANT MINERALS IN ALTERNATIVE DIETS FOR GILTHEAD SEA BREAM (*Sparus aurata*): BIOMARKERS, SOURCES AND ADEQUATE LEVELS

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(firma)	**(firma)**	**(firma)**

Las Palmas de Gran Canaria, a_____de_____de 20___

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α-act	Alpha-actin
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
ARA	Arachidonic acid
ATP	Adenosin triphosphate
ATP7a	ATPase 7a
ATP7b	ATPase 7b
Bact	Beta actin
BHT	Butylated hydroxytoluene
bmp	Bone morphogenetic protein
Ca	Calcium
cat	Catalase
Cd	Cadmium
cDNA	Complementary desoxiribonucleic acid
CEBA	Bioethical committee
Ct	Cycle threshold
Cu	Copper
$CuSO_4$	Copper sulphate
CuZnSOD	Copper zinc superoxide dismutase
ctr1	Copper transporter 1
C-	Negative control diet
DE	Encapsulated diet
DHA	Docosahexaenoic acid (22:6n-3)
DI	Inorganic diet

DO	Organic diet
DM	Dry matter
DMT1	Divalent metal transporter 1
dw	Dry weight
EFSA	European Feed Safety Authority
EPA	Eicosapentaenoic acid
EU	European Union
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FCR	Feed conversion ratio
Fe	Iron
FE	Feed efficiency
FM	Fish meal
FO	Fish oil
GIA	Aquaculture research group
Gmo	Genetically modified organism
GPx	Glutathione peroxidase
Gr	Glucocorticoid receptor
GSSG	Oxidized glutathione / glutathione disulphide
GSH	Reduced glutathione
H&E	Haematoxylin – eosin staining
HUFA	Highly unsaturated fatty acid
H_2O_2	Hydrogen peroxide
ICP-MS	Inductively coupled plasma mass spectrophotometry
IMR	Institute of Marine Research
K	Potassium
MCP	Mono calcium phosphate

MDA	Malonaldehyde
Mg	Magnesium
Mn	Manganese
MnAA	Amino acid chelated manganese
MnO_2/ MnOx	Manganese oxide
$MnSO_4$	Manganese sulphate
MnSOD	Manganese superoxide dismutase
mRNA	Messenger ribonucleic acid
Na_2SeO_3	Sodium selenite
ND	Not defined
NIFES	Norwegian Institute of Nutrition and Seafood Research
NRC	National Research Council
ns	Not significant
oc	Osteocalcin
$\cdot O_2^-$	Superoxide anion
$\cdot O_2^{-2}$	Peroxide
$\cdot OH$	Hydroxyl radical
OH^-	Hydroxyl ion
P	Phosphorus
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acids
rpl27	Ribosomal protein 27a
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Quantitative time real time PCR
SA	South American
SD	Standard deviation

Se	Selenium
SEP	Selenoproteins
SOD/*sod*	Superoxide dismutase
SGR	Specific growth rate
STD	Standard
TBARs	Thiobarbituric acid reactive substances
TGC	Thermal growth coefficient
ULPGC	University of Las Palmas de Gran Canaria
VL/FL	Vertebral length / standard length
VL/VH	Vertebral length / height
VM	Vegetable meal
VO	Vegetable oil
V13	Vertebra number 13
WG	Weight gain
WP	Work package
Zn	Zinc
ZnAA	Amino acid chelated zinc
ZnO/ ZnOx	Zinc oxide

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CHAPTER 1: INTRODUCTION

1.1. CURRENT STATUS OF AQUACULTURE

Since the decade of the 1950's, fish production, both derived from fisheries and aquaculture, has increased from 20 to 171 million tonnes (FAO, 2018). The greatest growth has occurred in the aquaculture sector, reaching an annual increment of 9.5% in 1990-2000. After some reduction due to the global economic crisis, production growth lowered to 5.8% for the period 2000-2016 (FAO, 2018). Nowadays, the aquaculture industry produces 53% of the world's consumed fish (FAO, 2018), whilst fisheries still have the upper hand due to the yield derived from fish not intended for direct human consumption (FAO, 2018).

The aquaculture industry in the EU is predominantly marine, with almost 78% of the total production, and being two of the three most cultured finfish species Atlantic salmon (*Salmo salar*) and gilthead sea bream (*Sparus aurata*) (APROMAR, 2018). Production areas and species are divided into three major locations: freshwater production, with trouts and carps; the North Atlantic Ocean, with salmon production; and the Mediterranean Sea, with gilthead sea bream and European seabass (*Dicentrarchus labrax*).

1.2. GILTHEAD SEA BREAM PRODUCTION

Gilthead sea bream is a teleost from the *Sparidae* family and has a compressed shape distinguishing it from other sparids thanks to the black spot that partially covers the opercula and the yellow band between its eyes. This species is distributed throughout the Mediterranean Sea, and in the Atlantic Ocean from the United Kingdom down to Cape Verde. It can be found in all kinds of seabeds, where it feeds upon molluscs, crustaceans and smaller fish, and can inhabit brackish waters and estuaries, due to its euryhaline and eurythermal condition (APROMAR, 2018).

Gilthead sea bream culture began at a large scale in the 1990's. Gilthead sea bream world production is focused in Europe and especially the Mediterranean Sea, where practically every country culture it. Spain represents the fourth sea bream producer globally with 13.6 thousand tonnes produced in 2017 (APROMAR, 2018).

For the further expansion of the production of this species, current research is focused on genetic selection, development of sustainable feeds replacing fish meal and oil (FM/FO), and the improvement of fish health through the use of functional feeds with high levels of omega 3, vitamins and minerals (Barazi-Yeroulanos, 2010).

1.3. NUTRITION AND INGREDIENTS

Feeds in the Spanish aquaculture industry stand for the 31% of the total production costs, meaning it is a major cost (Salz, 2009). Traditionally aquaculture feeds were produced based upon FM/FO which are limited resources, highly dependent on fisheries and with a tendency to rising prices due to the competence with other industries (Tacon and Metian, 2008; Tacon and Metian, 2009; Tacon and Metian, 2015). Substitution of FM/FO in fish feeds is currently one of the industry's priorities due to the increasing prices and lack of stability of the market. Vegetable meals and oils (VM/VO) can partially replace FM/FO in gilthead sea bream diets (Benedito-Palos *et al*., 2007; Gómez-Requeni *et al*., 2004; Izquierdo *et al*., 2005; Montero *et al*., 2003; Robaina *et al*., 1995), economically improving the efficiency of the production whilst generating a nutritive and secure product in a sustainable way. Nowadays, it is common to find dietary inclusion levels of plant ingredients up to 78% of the total feed for herbivorous and omnivorous fish, and around 45% for marine and carnivorous fish (Tacon and Metian, 2015). Common plant ingredients used in gilthead sea bream diets are derived from soybean, rapeseed,

sunflower, wheat, linseed and corn (ARRAINA, 2015). Therefore the EU has opted to invest in the substitution by vegetable ingredients as sources for proteins and lipids.

Nutritional composition of feeds based on marine ingredients differ significantly to those based upon vegetable ingredients, and can cause negative effects in growth, resistance to disease and productivity of the fish. Some of these negative effects are increased lipid deposition in the liver (Robaina *et al*., 1995) and reduced antioxidant status (Saera-Vila *et al.*, 2009), even when the feeds are formulated to contain the required amounts of essential amino acids and fatty acids (NRC, 2011), which suggests possible micronutrient imbalances. This can be due to different micronutrient levels between fish and vegetable ingredients (ARRAINA, 2015).

1.4. MINERALS IN FISH NUTRITION

Minerals are essential inorganic elements for all life processes in animals, including fish. They intervene in the formation of the skeleton, regulation of the acid-base equilibrium and maintaining the colloidal systems. Many minerals are also components of enzymes, metalloproteins and hormones. Minerals can be divided into macrominerals, those required in quantities above a gram, including calcium (Ca), magnesium (Mg), sodium, potassium, and chlorine; and microminerals, required in lower levels, including copper (Cu), iron (Fe), manganese (Mn), selenium (Se) and zinc (Zn) among others (Lall, 2002; Watanabe *et al*., 1997). Marine fish can obtain certain minerals from the water, whilst others must be included in the diet (Lall, 2002; NRC, 2011). Some of these minerals used to be included in enough quantities through fish derived ingredients, however, the increased substitution of these by vegetable ingredients has radically altered the dietary mineral balance.

Establishing nutritional requirements for a species is a fundamental step in order to maximize its productive potential. Several requirements for minerals have been established for species such as carps, catfish and salmonids (NRC, 2011). However, most of the requirements for gilthead sea bream are yet unknown, forcing the feed producers to extrapolate the requirements of other species to produce sea bream feeds, some of which can present different physiology. Therefore, there is a wide range of nutrients for which the requirement for sea bream is totally unknown.

As essential nutrients, mineral deficiencies may have deleterious effects on vital processes which ultimately translate in an impairment of function, thus impeding the organism to complete its life cycle (Lall, 2002). On the other hand, excess of these minerals may also alter the physiological processes by induced toxicity, due to both waterborne or dietary exposure to the mineral. The mechanism of action of this intoxication may vary and include the blockade of enzymatic groups, displacement of another metal ion in a biomolecule and modification of active sites of molecules (Lall, 2002).

Mineral sources are present in a wide range, from the ions present in the water to macromolecules such as amino acid complexes. Use of inorganic salts has been the main traditional source of minerals in the aquaculture industry. However, the availability of some of these salts may be low for certain minerals. Some amino acid chelates are able to deliver the mineral at the absorption site or directly absorb it as an intact chelate, thus reducing the loss of the element (Scott *et al*., 1982).

Mineral availability may be reduced by interactions with other minerals or by the presence of anti-nutrients in the feed (Olsen *et al*., 2007; Hansen and Hemre, 2013). For instance, certain complexes (anti-nutritional factors) present in vegetable meals, such as phytic

acid, act chelating minerals in the digestive tract, thus reducing its bioavailability (Francis *et al.*, 2001; Satoh *et al*., 2001).

1.5. ZINC (Zn)

1.5a. Function and metabolism

Zinc is an essential trace element for fish that intervenes in a wide series of metabolic processes. It is an integral component of more than 20 metalloenzymes and intervenes in structural and catalytic processes that imply over 300 proteins related with vital functions such as growth, reproduction, tissue development, vision and immunity. Many metabolic pathways involved in carbohydrate, lipid and protein metabolism are directly regulated by Zn, as it forms part of a battery of essential enzymes such as carbonic anhydrase, alkaline phosphatase, carboxypeptidases A and related peptidases. It also intervenes in regulating oxidative stress and reducing cellular damage by actively forming part of antioxidant enzymes including alcohol dehydrogenases, and cytosolic superoxide dismutase. Actually, in terms of quantity, Zn is the second major trace mineral in importance after Fe in the organism (Lall, 2002; Watanabe *et al*., 1997).

Zinc intervenes on bone formation and mineralization by activating osteoblastic cells and inhibiting osteoclastic bone resorption, hence promoting ossification, bone development and subsequently growth (Yamaguchi, 1998). Studies have demonstrated that Zn can partially substitute calcium in the bone due to their similar physic-chemical properties (Do Carmo e Sá *et al*., 2004).

Zinc assimilation occurs mainly at the gills and the intestine. In low quantities of dietary Zn, the gills play an important role in maintaining Zn intake, especially in freshwater species (Spry and Wood 1989). However the major absorption is in the intestine which

uptakes both waterborne and dietary Zn (Glover and Hogstrand, 2002; Lall, 2002; Spry and Wood, 1988; Watanabe *et al*., 1997; Zhang and Wang, 2007b). Recent studies on amberjack (*Seriola dumerili*) suggest that larvae can control mineral status by regulating Zn uptake and/or excretion of minerals in the feed (Yamamoto *et al*., 2013).

1.5b. Requirements

Dietary Zn requirements have been studied in a series of anadromous, catadromous and diadromous fish species and with a wide range of feeding habits, from strictly carnivorous fish (such as turbot, *Psetta maxima* and malabar grouper, *Epinephelus malabaricus*) to omnivores and herbivores (such as Nile tilapia, *Oreochromis niloticus* and grass carp, *Ctenopharyngodon idella*) (Table 1.1). The differences in feeding habits and the mineral composition of the water highly influences Zn absorption thus altering the dietary requirements (Alsop *et al*., 1999; Clearwater *et al*., 2002; Spry and Wood, 1989; Watanabe *et al*., 1997). The influence of waterborne minerals takes special relevance in marine fish, where fish drink seawater for osmoregulation, affecting mineral uptake (Clearwater *et al*., 2002).

Being so, 20 mg Zn kg^{-1} diet have been reported as the minimum requirement for optimum development in channel catfish (*Ictalurus punctatus*, Gatlin and Wilson, 1983), blue tilapia (*Oreochromis aureus*, McClain and Gatlin, 1988), yellow catfish (*Pelteobagrus fulvidraco*, Luo *et al*., 2011) and red drum (*Sciaenops ocellatus*, Gatlin *et al*., 1991). Purified/semi-purified diets were formulated for these experiments, where white egg and a blend of cod liver oil and vegetable oils (corn or soybean) was mostly used. Practical diets often incorporate a series of elements difficult to assess and which may interact with the target mineral, complicating the interpretation of the study (Clearwater *et al*., 2002). On the other side, when purified/semi-purified diets are used,

relative percentage of other minerals are much easier to control, hence reducing the levels of Zn chelators/competitors may reduce Zn requirements. This may be the reason why channel catfish fed basal practical diet required at 50-59 Zn mg/kg (Li and Robinson, 1996). The inherent Zn content of the ingredients in the practical diet was enough to cover the requirements. However, these requirements were higher than those previously described (20 mg Zn kg^{-1}, Gatlin and Wilson, 1983; 20.86 mg Zn kg^{-1},Luo *et al*., 2011) for this species, probably due to the interactions with other substances present in the diet with Zn absorption.

Dietary requirement for Zn in Nile tilapia has also been studied. Based upon bone Zn content dietary requirements of 79.51 mg Zn kg^{-1} have been described for this species (Do Carmo e Sá *et al*., 2004), higher than those required by a close species, the blue tilapia (20 mg Zn kg^{-1}; McClain and Gatlin, 1988), in relation to the use of vegetable ingredients in the practical diets of the first study. The presence of high levels of phytic acid in diets fed to Nile tilapia may have caused dietary Zn to chelate, thus reducing its uptake (Do Carmo e Sá *et al*., 2004).

The dietary requirements of Zn for carps have also been described in several species. For Jian carp (*Cyprinus carpio* var. *Jian*), Zn requirements when fed purified vegetable diets supplemented with Zn lactate were 48.69 mg Zn kg^{-1} (Tan *et al*., 2011a). This values are similar to those s stablished for grass carp fed semi-purified diets supplemented with $ZnSO_4$ (53 mg kg^{-1} Liang *et al*., 2012). This illustrate the difference in the definition of the required dietary levels when either semi-purified or practical diets are used.

Table 1.1 Dietary zinc requirements studied for different fish species

Species	Scientific name	Requirement (mg Zn kg^{-1})	References
Atlantic Salmon	*Salmo salar*	37–67[3]	Maage and Julshamn, 1993
Blue tilapia	*Oreochromis aureus*	20[3]	McClain and Gatlin, 1988
Channel catfish	*Ictalurus punctatus*	20[3]	Gatlin and Wilson, 1983
		Egg white: 6.58[Zn methionine 1, 3]	Paripatananont and Love, 1995
		Egg white: 19.91[ZnSO4 heptahydrate 1, 3]	Paripatananont and Love, 1995
		Soybean meal: 12.82[Zn methionine 1, 3]	Paripatananont and Love, 1995
		Soybean meal: >80[ZnSO4 heptahydrate 1, 3]	Paripatananont and Love, 1995
		50-59[1, 3, 5]	Li & Robinson 1996
Grass carp	*Ctenopharyngodon idella*	53[2, 3]	Liang *et al*., 2012
Guppy	*Poecilia reticulata*	80[1, 2]	Shim and Lee, 1993
Hybrid Striped Bass	*Morone chrysops* × *Morone saxatilis*	17.3[1, 3]	Buentello *et al*., 2009
Hybrid tilapia	*Oreochromis niloticus* × *O.aureus*	135[1, 2]	Zhao *et al*., 2011
Jian carp	*Cyprinus carpio var. Jian*	48.69[1, 2, 5]	Tan *et al*., 2011a
Malabar grouper	*Epinephelus malabaricus*	33.7[1, 2, 3, 5]	Houng-Yung *et al*., 2014
Nile Tilapia	*Oreochromis niloticus*	79.51[1, 2, 3]	do Carmo e Sá *et al*., 2004
Rainbow trout	*Oncorhynchus mykiss*	40[3]	Apines *et al*., 2001
Rainbow trout	*Oncorhynchus mykiss*	15–30[1, 3, 5]	Ogino and Yang, 1978
Red drum		20-25[3]	Gatlin *et al*,. 1991
Turbot	*Psetta maxima*	60.2[2, 3, 4, 5]	Ma *et al*., 2014
Yellow catfish	*Pelteobagrus fulvidraco*	20.86[1, 3, 5]	Luo *et al*., 2011

Criteria used to evaluate requirements: 1- Final weight/ weight gain, 2- Feed efficiency/ feed intake/ FCR, 3-Zn tissue retention, 4- Antioxidant activity markers (including TBARs, CuZnSOD, GPx, etc), 5-Other markers.

Interestingly, both salinity and mineral composition of the water affect Zn uptake. Such is the case in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*), two very related phylogenetic species living in different habitats. Zn requirements for rainbow trout are 15-30 mg Zn kg^{-1} (Ogino and Yang, 1978), whilst Atlantic salmon requires 37–67 mg Zn kg^{-1} (Maage and Julshamn, 1993). Studies on catadromous species include Asian seabass/barramundi (*Lates calcarifer*, 45 mg Zn kg^{-1}, Sapkale and Singh, 2011), cobia (*Rachycentron canadum*, 47-54 mg Zn kg^{-1}, Xu *et al*., 2007), European seabass (*Dicentrarchus labrax*, 240 mg Zn kg^{-1}, Fountoulaki *et al*., 2010), Malabar grouper (33.7 mg Zn kg^{-1}, Houng-yung *et al*., 2005), hybrid stripped bass (*Morone chrysops* × *Morone saxatilis*, 17 mg Zn kg^{-1}, Buentello *et al*., 2009), Japanese seabass (*Lateolabrax japonicus*, 103 mg Zn kg^{-1}, Zhou *et al*., 2009), red drum (20 mg Zn kg^{-1}, Gatlin *et al*., 1991) and turbot (60.2 mg Zn kg^{-1}, Ma *et al*., 2014). The results from these trials show the large variations for Zn requirements between the different marine species, despite their similar feeding habits.

1.5c. Deficiency and toxicity

As Zn intervenes in such a wide variety of metabolic processes, deficiencies also imply the alteration of different physiological mechanisms and deficiency effects become inespecific and severe. Zinc deficiency in fish causes slower growth rate, cataracts, high mortality, anorexia, low tissue Zn, skin and fin erosion or dwarfism (Ogino and Yang; 1978; Watanabe *et al*. 1997). Also, increased bone anomalies and reduced DHA content have been related to Zn deficiency in red sea bream (*Pagrus major*, Nguyen *et al*., 2008).

Metal uptake and toxicity in marine fish are usually much lower than those in freshwater fish (Zhang and Wang, 2007a). However toxicity in freshwater fish species has been described, especially for rainbow trout, which seems much more sensible to this mineral

than other fish species. Probably the most important toxic effect of Zn is related to the inhibition of calcium absorption at high levels of Zn. Calcium and Zn share transport channels, and at high levels of Zn, calcium uptake is severely reduced (Hansen *et al*., 2002; Hogstrand *et al*., 1996). Zinc can partially replace calcium in the bones due to their similar properties, therefore disrupting bone morphology (do Carmo e Sá *et al*., 2004). Therefore excess of Zn may inhibit calcium intake, thus altering calcium-dependant metabolic pathways such as nerve signaling, promoting calcium liberation from the bone and thus, reducing bone strength (Hogstrand *et al*., 1996). High inclusion of dietary Zn may also be related to a decrease in Fe in bones (Signor *et al*., 2010). This may be another possible toxic effect of Zn in the diets, which has been scarcely studied.

Relative Zn toxicities have been studied for some freshwater species, whereas toxicity in marine fish is yet to be study. Some studies described mineral uptake and excretion regulation of Zn in *Seriola dumerili* larvae (Yamamoto *et al*., 2013). This, combined with the lower bioavailability of Zn in marine water may increase the threshold at which Zn produces toxic effects on marine fish. Even though, toxicities in freshwater fish have been studied mainly for waterborne Zn, whereas in marine fish both waterborne and dietary Zn toxicity ranges are yet to be stablished.

1.5d. Sources

The source/chemical formulae in which Zn is supplemented to the diet may be decisive for Zn uptake. Fish absorb Zn via the gills (waterborne) and the intestine (waterborne and dietary) (Lall, 2002; Spry and Wood, 1988; Watanabe *et al*., 1997). Dietary Zn uptake in the intestine seems to be more important than waterborne in the gills, were total Zn absorption at long term (after 4h) can even be neglected when compared to dietary Zn (Zhang and Wang, 2007b). However, at low Zn levels, waterborne Zn can be fundamental

for uptake in fish both at the intestine (Glover and Hogstrand, 2002a) and at the gills (Spry and Wood, 1988).

Bioavailability of several sources has been studied in different species. Inorganic sources such as Zn sulphate mono- and heptahydrate are widely used, but novel nutrient delivery factors such as amino acid chelates, organic or glass-embedded are being studied to increase bioavailability Zn in diets. When inorganic and organic sources are compared, a series of considerations have to be taken. To begin, gastrointestinal system of fish vary widely among species. Carnivorous species tend to have large stomachs (increasing the hydrolyzing effect on Zn due to the presence of HCl) but small intestines, whilst herbivorous species usually have small and large intestines. These morphological differences translate in different enzymatic activities, pH, and intestinal motilities, affecting both availability and absorption of Zn (do Carmo e Sá, *et al*. 2005; Glover and Hogstrand, 2002a, 2002b).

Zn bioavailability is also affected by the ingredient composition of the diet. The presence of a series of chelators or competitors diminishes Zn bioavailability. Phytic acid is one of the most important metal chelators present in vegetable ingredients. Phytic acid forms insoluble Zn phytates in the intestinal lumen, reducing Zn uptake (Apines-Amar *et al*., 2004; Watanabe *et al*., 1997). This is especially true for most inorganic sources, which are more susceptible to being chelated by phytic acid (Clearwater *et al*., 2002; Lall, 2002; Paripatananont and Lovell, 1995), whilst amino-acid chelated Zn seem to increase Zn bioavailability (Apines *et al*., 2001; Savolainen and Gatlin, 2010). Thus, the presence of phytic acid reduces ZnO uptake in relation to an impeded hydrolization of ZnO by the HCl present in the stomach (Do Carmo e Sá *et al*., 2005). The use of purified or even semi-purified diets implies a reduction in the levels of chelates and competitive molecules. Therefore, different studies conducted in channel catfish fed purified egg-

white based diets with $ZnSO_4$ presented similar Zn requirements (20 mg Zn $kg^{-1,}$ Gatlin and Wilson, 1983 and 19.91 mg Zn kg^{-1}, Paripatananont and Lovell 1995). In this case very similar diets were used, however when compared to the use of soybean diets, the requirements were much lower for Zn methionine (12.82 mg Zn kg^{-1}) than for Zn sulphate (>80 mg Zn kg^{-1}). In this study, Zn methionine offered a higher bioavailability probably in relation to the fact that sulphates form insoluble phytates in the presence of phytic acid, whilst amino acid chelates are more readily absorbed (Apines-Amar *et al*., 2004).

Calcium also plays an important role in Zn uptake. Several studies suggest that high dietary Ca levels decrease Zn uptake (Apines-Amar *et al*., 2004; do Carmo e Sá, 2005; Lall, 2002). Ca also seems to reduce waterborne Zn uptake via the gills (Barron and Albeke, 2000; Clearwater *et al*., 2002; Spry and Wood, 1989). Furthermore, Ca seems to share transport channels with Zn in the gills (Hansen *et al*., 2002; Hogstrand *et al*., 1996). Other studies suggest high Zn levels can partially substitute Ca from bone due to their similar physic-chemical properties (do Carmo e Sá *et al*., 2004). However Ca levels in diets are usually controlled, the problem arises when waterborne Ca (in its carbonate forms $CaCO_3$) is not taken into consideration. In such cases water salinity increases and Ca competes directly with waterborne Zn, saturating the transport channels and decreasing Zn uptake (Zhang and Wang, 2007a). Therefore salinity plays also an important role in Zn absorption, meaning lower salinities increase Zn uptake capacity (De Schamphelaere and Janssen, 2004; Zhang and Wang, 2007a). This may explain why dietary Zn requirements for rainbow trout (Ogino and Yang, 1978) are lower than for Atlantic salmon (Maage and Julshamn, 1993). Freshwater rainbow trout live in a habitat with lower levels of waterborne Ca, whilst marine water Atlantic salmon have reduced Zn uptake via the gills due to the high concentration of waterborne Ca, meaning dietary Zn requirements must be higher to compensate this lower Zn availability.

pH can affect waterborne Zn uptake via the gills. High levels of H^+ in acid waters decrease Zn absorption due to direct competition with Zn transport channels (Alsop and Wood, 1999; Hansen *et al*., 2002) or by damaging these channels (Alsop and Wood, 1999).

1.5e. Biomarkers

Serum Zn values, body, vertebrae and *cuznsod* expression are among the biomarkers used to establish Zn requirements (Antony Jesu Prabhu *et al*., 2016; Apines-Amar *et al*., 2004; Gatlin, 1983; Paripatananont and Lovell, 1995).

1.6. MANGANESE (Mn)

1.6a. Function and metabolism

Mn is a transition metal essential for life and acts as a cofactor for essential metalloenzymes involved in several enzyme complexes including the Mn superoxide dismutase (MnSOD) (Holley *et al*., 2011), but also intervenes in carbohydrate, lipid, and protein metabolism (Lall, 2002). As its chemistry is very similar to that of magnesium (Mg), Mg and Mn can often be used interchangeably by many enzymes (Lall, 2002). MnSOD is one of the three SODs found in vertebrates, and it is responsible for dismutation of reactive oxygen species (Giulio and Meyer, 2008), being an essential part of the antioxidant defence system of the cell (Holley *et al*., 2011). MnSOD is mainly associated to mitochondrion (Giulio and Meyer, 2008), which is vital for many metabolic pathways in the cell, contributing for diverse functions such as β-oxidation of fatty acids, the urea cycle, the citric acid cycle, and adenosine triphosphate (ATP) synthesis. Thus, mitochondrion is also a major site of reactive oxygen species production in the cell (Giulio and Meyer, 2008).

Water dissolved Mn can be absorbed by fish as well. However, diet borne Mn is the main source of uptake due to the presence of calcium and phosphorus in the water, which reduce its availability (Watanabe *et al*., 1997). Mn is absorbed through the gut by passive diffusion or active transport, mainly by the divalent metal transporter 1 (DMT1) (Figure 1), however its transporters are not Mn-specific, but also regulate Fe, Cu, Zn and other metals uptake. Therefore, the presence of these metals will compete with Mn absorption (Chen *et al*., 2018). After being absorbed in the gut or the gills it distributes to the different tissues. Blood, specifically erythrocytes, plays an important role in Mn transport since it has the ability to transport it using Mn transporters, such as DMT1. The liver is the main organ able to regulate Mn levels through the expression of Mn transporters, including DMT1, on the cell membranes of hepatocytes. When an excess of Mn is detected, the liver gathers Mn and excretes it through the bile, being this the main excretion pathway of the mineral (Figure 1). Bone is another important Mn storage tissue, accounting for up to 40% of total Mn in humans. Other tissues with high levels of this mineral are liver, muscle, kidney, gonadal tissues, and skin, where it is more concentrated in the mitochondria (Chen *et al*., 2018; Lall, 2002).

Traditional fish feeds were formulated based on FM, however, the presence of large amounts of calcium and phosphorus present in these ingredients appear to reduce the availability of Mn present in diets containing white fish meal, meaning these diets had to be supplemented with additional Mn (Satoh *et al*., 1991). The use of alternative plant ingredients in modern day aquaculture feeds can lead to an increase in the Mn levels, due to its larger presence in plant ingredients than in FM (Sanden *et al*., 2017).

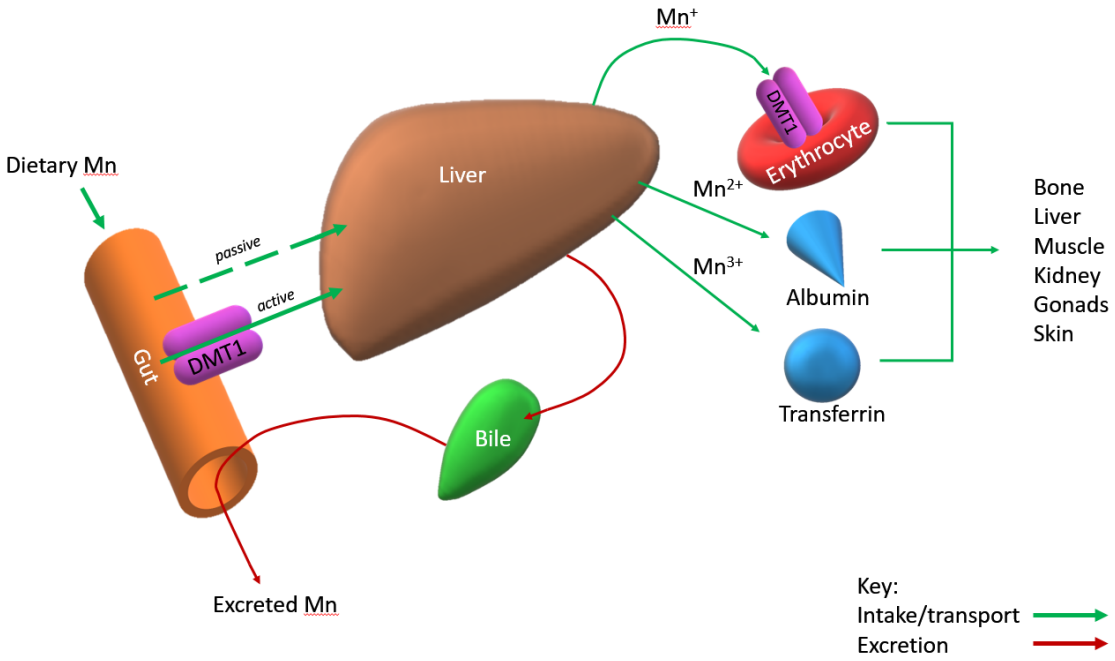

Figure 1 Proposed model of manganese homeostasis in vertebrates. Dietary manganese is absorbed in the gut through passive diffusion or by active transport by DMT1 to the liver. From the liver it is transported in the bloodstream to the different tissues. Mn^{+} is incorporated into the erythrocytes by DMT1, and transported. Mn^{2+} and Mn^{3+} are transported by albumin and transferrin respectively. Excess Mn is excreted to the gut through the bile (Adapted from Juttukonda and Skaar, 2017).

1.6b. Requirements

Studies on Mn requirements have been conducted on carnivorous, such as cobia, and herbivorous fish, such as carps. Similarly, freshwater and marine species have been studied. It seems as though marine species tend to have higher Mn requirements than freshwater species, although this is not always the case. Mn requirements range from as low as 2.4 mg Mn kg^{-1} for channel catfish (Gatlin and Wilson, 1984) to 21.72 mg Mn kg^{-1} for cobia (Liu *et al.*, 2013) (Table 1.2).

Table 1.2 Dietary manganese requirements studied for different fish species

Species	Scientific name	Requirement (mg Mn kg^{-1})	References
Atlantic salmon	*Salmo salar*	7.5-10.5[3]	Maage *et al*., 2000
		15[3]	Lorentzen *et al*., 1996
Cobia	*Rachycentron canadum*	21.72[1, 2, 3, 4]	Liu *et al*., 2013
Common carp	*Cyprinus carpio*	13-15[1, 3]	Satoh *et al*., 1992
Channel catfish	*Ictalurus punctatus*	2.4[1, 2, 3, 4, 5]	Gatlin and Wilson, 1984a
Gibel carp	*Carassius auratus gibelio*	13.77[1, 2, 3, 4]	Pan *et al*., 2008
Hybrid tilapia	*Oreochromis niloticus X O. aureus*	7[3, 4]	Lin *et al*., 2008a
Orange-spotted grouper	*Epinephelus coioides*	19[2, 3]	Ye *et al*., 2009
Hybrid grouper	*Epinephelus lanceolatus* × *E. fuscoguttatus*	12.7[1, 2, 3, 4, 5]	Liu *et al*., 2017
Rainbow trout	*Oncorhynchus mykiss*	19[1, 3, 5]	Satoh *et al*., 1991
Yellow catfish	*Pelteobagrus fulvidraco*	5.5-6.4[1, 2, 3, 4, 5]	Tan *et al*., 2012

Criteria used to evaluate requirements: 1- Final weight/ weight gain, 2- Feed efficiency/ feed intake/ FCR, 3-Mn tissue retention, 4- Antioxidant activity markers (including TBARs, MnSOD, GPx, etc), 5-Other markers.

1.6c. Deficiency and toxicity

An insufficient Mn supplementation usually translates into an inefficient function of the metabolic processes in which Mn is involved. MnSOD activity is reduced in the heart and liver, as well as the level of Mn, Ca and Na 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). Bone formation can be severely hampered in fish fed a diet deficient in Mn (Nguyen *et al*., 2008).

Mn toxicity risk appears to be low. Orange-spotted grouper (*Epinephelus coioides*) fed very high levels of Mn (1000 mg Se kg^{-1}) presented reduced feed efficiency and survival, however these levels are well beyond the levels supplemented in traditional feeds (Ye *et al*., 2009).

1.6d. Sources

Several sources of Mn are currently used in the industry. Inorganic salts such as sulphates ($MnSO_4$), oxides (MnO_2) and chlorides ($MnCl_2$) are commonly used, and the source employed varies from species to species, for instance salmon are usually fed with sulphates (Maage *et al*., 2000). On the other hand, amino acid chelates appear to be more effective for rainbow trout (Apines-Amar *et al*., 2004). However, a consensus has not been still reached regarding which is the most suitable source of Mn.

1.6e. Biomarkers

Despite a deficiency in Mn can translate in a reduction in growth and feed efficiency, the truth is that these are not ideal biomarkers to assess Mn requirements. The most reliable criteria for Mn requirements are tissue Mn, especially vertebral Mn, and to a lesser extent MnSOD activity (Antony Jesu Prabhu *et al*., 2016). Other biomarkers such as the level of Mn in heart and whole body content are also used as Mn biomarkers (Knox *et al*., 1981; Maage *et al*., 2000).

1.7. SELENIUM (Se)

1.7a. Function and metabolism

Selenium (Se) is a non-metal element considered essential due to its involvement in the production of thyroid hormone and the proper functioning of the immune and reproductive systems (Rayman, 2000). It also plays an important role in the reduction of oxidative stress by forming part of the selenoproteins (SEP), including glutathione peroxidase (GPx) where it is a functional component of the active site (Wendel *et al*., 1975).

Aerobic respiration and other metabolic processes involving oxygen consumption produce reactive oxygen species as a byproduct. These unstable free radicals are a potential cause of oxidative damage to proteins, DNA and lipids. Among these we can find superoxide anions ($\cdot O_2^-$), peroxides ($\cdot O_2^{-2}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$) and ions (OH^-) (Apel and Hirt, 2004). Marine fish species tissues, such as gilthead sea bream, contain high levels of (n - 3) series unsaturated fatty acids that are particularly susceptible to oxidative damage. To counter-act the formation of these harmful compounds, vertebrates possess two major enzyme systems: catalases and peroxidases (including GPx) (Di Giulio and Meyer, 2008; Holley *et al*., 2011). Whereas the latter can act on various organic peroxides, including H_2O_2, catalases are widely restricted to H_2O_2. GPx is an ubiquous peroxidase involved in the reduction of a peroxide to its corresponding alcohol, coupled with the oxidation of reduced glutathione (GSH) to glutathione disulphide (GSSG). In the case of H_2O_2, the corresponding alcohol is water: $H_2O_2 + 2GSH \rightarrow GSSG + H_2O$ (Figure 2). Whenever the pro/anti-oxidant balance favours the pro-oxidation, the organism enters in a status named oxidative stress (Giulio and Meyer 2008). Teleost fish are the organisms with the highest number of SEP (Lobanov *et al*., 2009; Mariotti *et al*., 2012), and four GPx isoforms have been described in gilthead

sea bream (Malandrakis *et al*., 2014). Thus, Se as a component of GPx is an important factor in cellular defence mechanisms against oxidative damage (Bell *et al*., 1987). The other enzymatic system 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 these organelles. Thus catalase protects the peroxisomes and prevents the expansion of H_2O_2 to other locations, but mainly restrains to this organelle (Di Giulio and Meyer, 2008).

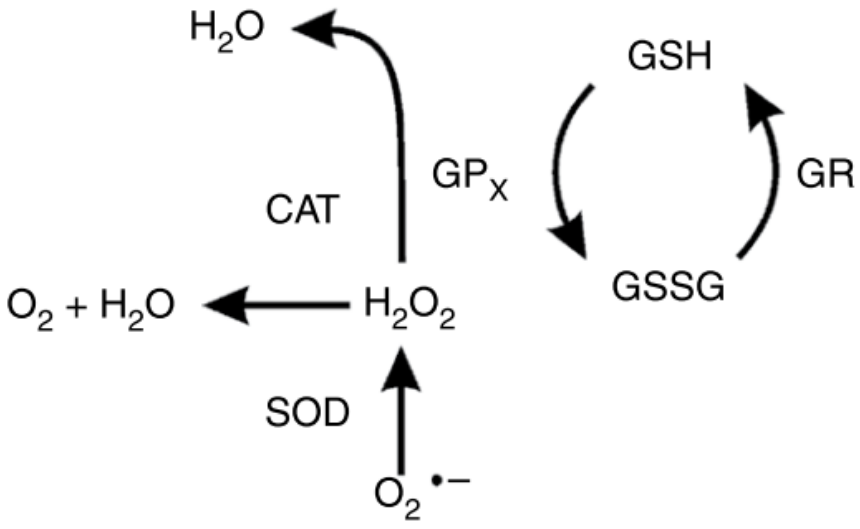

Figure 2. The three main intracellular antioxidant enzymes in fish are—superoxide dismutases (SOD), catalase and peroxidase,. The SODs convert $O_2^{\bullet -}$ into H_2O_2, wh the catalases (CAT) and peroxidases (GPx) convert H_2O_2 into water. GPx requires several secondary enzymes, including glutathione reductase (GR), and cofactors, including glutathione (GSH), and glutathione disulfide (GSSG) (Adapted from Weydert and Cullen, 2010).

Oxidative stress appears to alter the activity of transcription factors by altering the redox status of the cell. Transcription factors in eukaryotes reported to be affected by ROS include glucocorticoid receptors (*gr*) (Arrigo, 1999; Di Giulio & Meyer 2008, Esposito

et al., 1998, Olsvik *et al*., 2011). These are Zn-finger containing proteins susceptible to inhibition by reducible Se compounds (with oxidation state of –I or higher), in which sodium selenite (NaSe) is included (oxidation state +IV) (Blessing *et al*., 2004). Furthermore, selenite catalyses the oxidation of sulfhydryl (SH) groups, such as those present in the glucocorticoid receptor hormone binding sites (Tashima *et al*., 1989).

In commercial aquafeeds for carnivorous fish, such as gilthead sea bream, 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 decades. 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 *et al*., 2017).

Fish can absorb Se both from the water and diet, even covering some species' requirements by absorbing water Se (Hodson and Hilton, 1983). Se is mainly absorbed in the gut, where it is transported to the liver through the blood, and then reduced to selenide (H_2Se), before being transported in the blood, to various organs and tissues. Selenide is considered the central point in the interconversions of organic and inorganic Se compounds (Fairweather-Tait *et al*., 2011). Se is incorporated into specific selenoproteins, as selenocysteine, and, nonspecifically, as selenomethionine. Dietary selenomethionine is converted to selenocysteine or incorporated into proteins in place of methionine. Selenate can transform into selenite, and can be reduced to selenide directly, or it can react with glutathione to form selenodiglutathione, a substrate for glutathione reductase (Figure 3) (Fairweather-Tait *et al*., 2011).

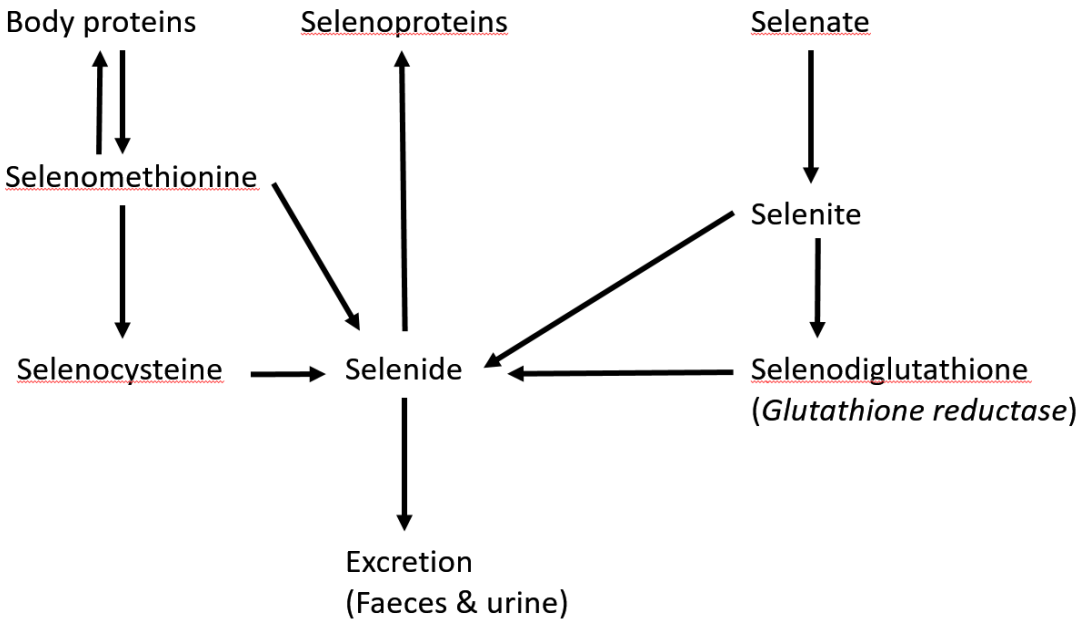

Figure 3. Selenium metabolism scheme. The scheme shows the pathways through which dietary selenium obtained as selenocysteine, selenomethionine, selenate, and selenite, are converted to selenide (H_2Se), and how this intermediate is incorporated into selenoproteins as selenocysteine or enters the excretion process (adapted from Fairweather-Tait *et al.*, 2011).

The main tissues for Se deposition are red blood cells, liver, spleen and heart. After its incorporation, Se is withdraw from the tissues with relative ease, mainly via urine, except for plasma, kidney and liver, where it remains for some time (Hodson and Hilton, 1983; Reilly, 2006).

1.7b. Requirements

Se nutrition in fish species has received a considerable attention during the last years, thus requirements studies have been conducted on the main groups of fishes cultured. Furthermore, both carnivores and herbivores, and marine and freshwater species have been studied. Studies on Se requirements have the additional handicap of the importance of the source, hence a number of studies have been conducted on the same species with different sources. Overall, the range of Se requirements goes from 0.12 mg Se kg^{-1} for

channel catfish, a species particularly sensible to Se intoxication, to 7.37 mg Se kg^{-1} for yellowtail kingfish (*Seriola lalandi*) (Table 1.3). Se 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).

Table 1.3 Dietary selenium requirements studied for different fish species

Species	Scientific name	Requirement (mg Se kg^{-1})	References
Atlantic salmon	*Salmo salar*	≤1.0-2.0 sodium selenute 1, 3, 4, 5, 6	Berntssen *et al*., 2018
		≤2.8 L-selenomethionine 1, 2, 3, 4, 5, 6	Berntssen *et al*., 2018
		0.42 Parr, freshwater* 1, 4, 5, 6	Hamre *et al*., 2016; Hemre *et al*., 2016
		0.45 Post-smolt, sea* 4, 5	Hamre *et al*., 2016; Hemre *et al*., 2016
		<1.2 1, 4	Lorentzen *et al*., 1994
		0.94 1. 2, 3, 4, 5, 6	Bell *et al*., 1987
African catfish	*Clarias gariepinus*	0.3 † 1, 2, 4, 5, 6	Abdel-Tawwab *et al*., 2007
Barramundi	*Lates calcarifer*	3.5-4.5 1, 2, 4, 5	Ilham *et al*., 2016
Channel catfish	*Ictalurus punctatus*	0.2-0.3 sodium selenute 1, 2, 4	Wang and Lovell, 1997
		0.12 selenomethionine 1, 2, 4	Wang and Lovell, 1997
		0.12 selenoyeast 1, 2, 4	Wang and Lovell, 1997
		0.25 1, 2, 3, 4	Gatlin and Wilson, 1984b

Table 1.3 *Continued* Dietary selenium requirements studied for different fish species

Cobia	*Rachycentron canadum*	0.79-0.81[2, 3, 4, 6]	Liu *et al*., 2010
Common carp	*Cyrpinus carpio*	1.46 [1, 4]	Ashouri *et al*., 2015
		0.12[1, 2, 6]	Gaber, 2009
Cutthroat trout	*Oncorhynchus clarki bouvieri*	ND[1, 2, 3, 6]	Hardy *et al*., 2010
Gibel carp	*Carassius auratus gibelio*	1.18 [1, 2, 3, 4]	Han *et al*., 2011
Grass carp	*Ctenopharyngodon idellus*	0.73[3], 1.12[4], and 1.19[4]	Zhu *et al*. 2016
		0.83 [1, 2, 4, 5]	Liu *et al*., 2018
		0.55-0.60 [1, 2, 5]	Zheng *et al*., 2018
Hybrid striped bass	*Morone chrysops* × *M. saxatilis*	1.2 sodium selenite 1	Jaramillo *et al*., 2009
		0.9 selenomethionine 1	Jaramillo *et al*., 2009
		1.61 selplex® 1, 6	Cotter *et al*., 2007
		1.81 sodium selenute 1, 2, 4, 6	Cotter *et al*., 2007
Largemouth bass	*Micropterus salmoide*	1.60-1.85 [1, 4]	Zhu *et al*., 2012
Loach	*Paramisgurnus dabryanus*	0.48-0.50 [4, 5, 6]	Hao *et al*., 2014
Malabar grouper	*Epinephelus malabaricus*	0.90sodium selenute 1, 2, 6	Lin, 2014
		0.98selenomethionine 1, 2, 6	Lin, 2014
Meagre	*Argyrosomus regius*	0.7-0.77 [1, 2, 3]	Lin and Shiau, 2005

Table 1.3 *Continued* Dietary selenium requirements studied for different fish species

Nile tilapia	*Oreochromis niloticus*	1.06 [1, 2]	Lee *et al*., 2016
		1.5[4]	Atencio *et al*. 2009
Olive flounder	*Paralichthys olivaceus*	<4.13 [1, 2, 6]	Lee *et al*., 2010
Rainbow trout	*Oncorhynchus mykiss*	4.36 [1, 2, 4]	Wang *et al*., 2018a
		ND [sodium selenite 1, 2, 3, 4]	Küçükbay *et al*., 2009
		ND [selenomethionine 1, 2, 3, 4]	Küçükbay *et al*., 2009
		0.73 [basal] – 2.0 [under stress 3, 4, 5]	Rider *et al*., 2009a
Red sea bream	*Pagrus major*	1.34 [4, 5]	Dawood *et al*., 2019
Yellowtail kingfish	*Seriola lalandi*	5.56 [1, 4, 5, 6]	Le and Fotedar, 2013
		5.39-7.37 [1, 3, 4, 5]	Le and Fotedar, 2014a
		4.91 [1, 4, 5, 6]	Le and Fotedar, 2014b
White sturgeon	*Acipenser transmontanus*	<20.5 [1, 6]	Tashjian *et al*., 2006

Criteria used to evaluate requirements: 1- Final weight/ weight gain, 2- Feed efficiency/ feed intake/ FCR, 3- Se tissue retention, 4- Antioxidant activity markers (including TBARs, GPx, GR, others), 5- Immune response/haematology, 6- Other markers. *within a multiple nutrient package. †as supplemented. *ND*: not defined.

1.7c. Deficiency and toxicity

Se deficiency may cause reduced growth, mortality, lethargy, diminished appetite, muscle dystrophy, reduced vitamin E levels and low haematocrit (Watanabe *et al*., 1997). Se and vitamin E deficiency appear to be the main cause for the Hitra disease observed in salmon,

which presented severe alterations including muscular dystrophy and myocardial degeneration, anaemia and haemorrhages (Poppe *et al*., 1986). However, these severe symptoms are rare, and usually Se deficiency tends to curse with a more subtle symptomatology. One of the main effects of a deficiency in Se is the reduction of GPx activity, with the consequent increase oxidative risk that leads to a weakened immunity status (Khan *et al*., 2017).

Traditionally, Se has been more studied than other micro-minerals due to the potential toxicity of high concentrations. In fact, Se intoxication, known as "selenosis" is characterized by gastrointestinal disturbance, hair loss, fatigue and mild nerve damage in humans, while livestock undergo alkali disease, a condition that can be lethal (Pacitti *et al*., 2015). Se toxicity occurs when Se associates with sulphur-containing amino acids, thus altering functional enzymatic structures (Khan *et al*., 2017). This leads to a series of alterations including reduced growth and feed efficiency, increased oxidative stress and mortality, elevated skeletal anomalies, edema, 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 hematocrit (Bell *et al*., 1987; Berntssen *et al*., 2018; Betancor *et al*., 2012; Choi *et al*., 2015; Gatlin, 1983; Lin and Shiau 2005; Pacitti *et al*., 2013; Saleh *et al*., 2014; Schultz and Hermanutz, 1990; Tashjian *et al*., 2006; Watanabe *et al*., 1997; Zee *et al*., 2016b). Furthermore, under stressful conditions, Se requirements are increased, whereas under normal conditions these levels would be considered toxic (Khan *et al*., 2017). Se toxicity may also be influenced by the source of supplementation, where selenite can produce cytotoxicity by inducing oxidative stress in fish due to the production of hydrogen selenide that reacts with oxygen to produce reactive oxygen species (Misra *et al*., 2012).

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. 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).

1.7d. Sources

Se occurs naturally in foods and feedstuffs in organic complexes, primarily in the form of selenomethionine, selenocystine and selenocysteine. Fish meals and marine byproducts represent the best natural sources of Se among the common feedstuffs for fish (Lall, 2002). Nevertheless, Se derived from FM appears to have low digestibility when compared to other sources, being selenomethionine the more digestible, followed by selenite and selenocystine (Bell and Cowey, 1987).

All forms of Se, organic as well as inorganic, are readily absorbed. However, there are differences between levels of absorption, as well as of utilization, of the different chemical forms. In general, organic compounds, such as selenomethionine, are absorbed more efficiently than are inorganic forms, particularly selenite (Wang and Lovell, 1997). It has been argued that the reason behind the better bioavailability of organic Se than the inorganic Se is that unlike the selenite, selenomethionine is more efficiently integrated into fish tissues (Cotter *et al*., 2008; Lorentzen *et al*., 1994; Rider *et al*., 2009; Wang and Lovell 1997). In fact, only selenomethionine can be incorporated into proteins, being the storage form of Se mainly in the skeletal muscle. In contrast, NaSe can be incorporated into active selenoproteins, such as GPx, but not into selenomethionine as storage protein in liver and muscle (Rider *et al*., 2009).

1.7e. Biomarkers

Several criteria have been used to evaluate Se requirements for fish including weight gain, feed efficiency and other productive parameters (Table 1.3). The GPx homologues have been studied in gilthead sea bream (Malandrakis *et al*., 2014), and its activity and expression of glutathione peroxidase genes, including 1a (*gpx1a*) gene, have been used to evaluate Se requirements in fish species as one of the most responsive criteria (Antony Jesu Prabhu *et al*., 2016; Khan *et al*., 2017). Expression of catalase gene (*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 *et al*., 2011; Mansour *et al*., 2017; Misra *et al*., 2012; Penglase *et al*., 2014).

Se concentration in different tissues has also been employed as a criterion to evaluate Se absorption. Such is the case of liver Se, which follows a linear relation with dietary Se in a wide range of fish species including Atlantic salmon (Berntssen *et al*., 2018), channel catfish (Wang and Lovell, 1997), cobia (Liu *et al*., 2010), gibel carp (*Carassius gibelio*, Han *et al*., 2011), hybrid striped bass (Cotter *et al*., 2007), malabar grouper (Lin, 2014), Nile tilapia (Lee *et al*., 2016), olive flounder (*Paralichthys olivaceus*, Lee *et al*., 2010), rainbow trout (Hilton and Hodson, 1983; Wang *et al*., 2018a) or white sturgeon (*Acipenser transmontanus*, Tashjian *et al*., 2006). Se concentration in plasma has been also used as an indicator of Se status in vertebrates (Rayman *et al*., 2008).

Bone morphogenetic proteins (BMP) and osteocalcin (OC) are a group of proteins involved in bone development and intervene in a series of reactions that ultimately lead to osteoblast differentiation and ulterior osteogenesis in fish (Smith *et al*., 2006). Expression of their respective genes (*bmp* and *oc*) is considered an important biomarker of bone differentiation and mineralization, being, for instance, positively correlated to Se inclusion (Saleh *et al*., 2014). The level of *gpx-1* transcription is also considered as a sensitive biomarker for Se absorption (Pacitti *et al*., 2013).

1.8. COPPER (Cu)

1.8a. Function and metabolism

Cu is an essential metal involved in several Cu dependent enzymes that intervene in the defence against oxidation reactions and include the Cu/Zn superoxide dismutase (CuZnSOD). Cu also takes part in the production of energy at cellular level (through the C cytochrome oxidase), in neurotransmission, collagen synthesis and melanin production (Lall, 2002).

Superoxide dismutases (SOD) are part of the antioxidant systems enzymes involved in coping with reactive oxygen species produced as byproduct of aerobic metabolism. They accelerate the dismutation of superoxide anions ($\cdot O_2^-$) through the following reaction:

$\cdot O_2^- + \cdot O_2^- \rightarrow H_2O_2 + O_2$

Vertebrates posses three forms of SOD: CuZnSOD, which mainly occurs in the cytosol; MnSOD, which is highly associated with mitochondria; and ECSOD, an extracellular SOD which is also a CuZnSOD with a different molecular weight, and only present in mammals (Giulio and Meyer, 2008). Cu is thus, an essential component of CuZnSOD.

Fish can uptake Cu from the water or diet and usually builds up in eyes, liver, heart and brain (Bury *et al*., 2003; Lall, 2002; Watanabe *et al*., 1997). Cu absorption primarily takes place in the mid posterior region of the gut in fish (Handy *et al*., 2000). This absorption is related to dietary Cu, and, thus, efficiency of absorption is enhanced with low Cu status and repressed in the presence of high Cu status (Clearwater *et al*., 2000; Handy *et al*., 2000). Cu absorption at the intestinal level takes place via copper transporter 1 (Ctr1), DMT1 and endocytosis, while Cu transporting ATPase ATP7A is in charge of delivering this Cu into the secretory Cu proteins that will transport it to the liver (Prohaska, 2009; Collins, 2017, Figure 4). Absorbed Cu is then transported to the liver, where hepatocytes utilise it for metabolic needs and also synthesize ceruloplasmin, the major Cu containing protein in serum. Hepatocytes also cope with the excretion of excess Cu through the

canalicular membrane into the bile. Both the production of ceruloplasmin and the removal of excess Cu are performed by the Cu transporting ATPase ATP7B (Burkhead and Lutsenko, 2013; Collins, 2017). In gilthead sea bream fed diets high in Cu, hepatic *atp7b* mRNA expression was elevated by 3-fold after 15 days, suggesting that hepatic *atp7b* may also be transcriptionally regulated in a chronic response to dietary Cu-overload (Minghetti *et al*., 2010).

At a tissue level, Cu distribution is not homogenous. For instance, in rockfish (*Sebastes schlegeli*), the liver is a more important storage tissue than other tissues, and the order of Cu accumulation in tissues is liver > intestine > kidney > gill > muscle (Kim and Kang, 2004).

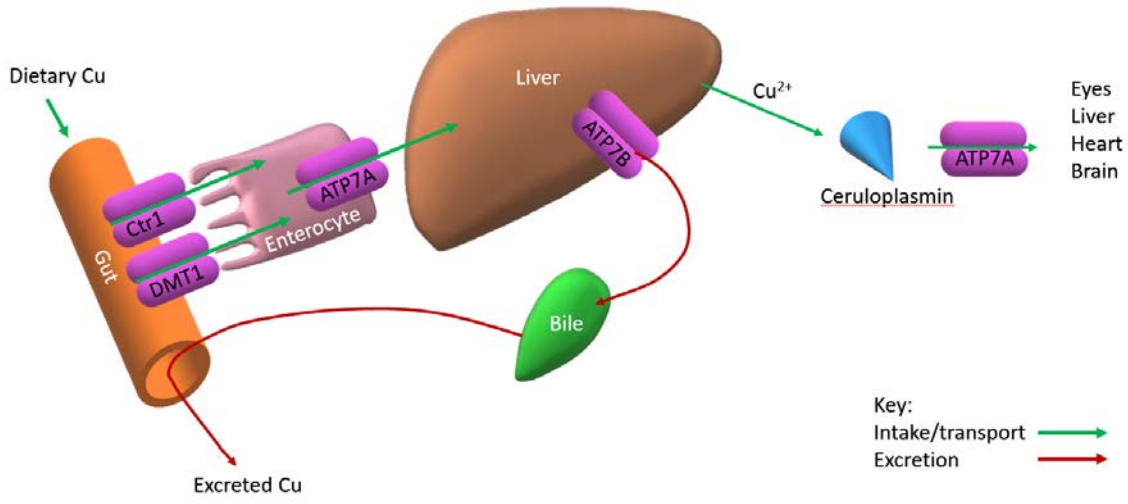

Figure 4. Simplified scheme of copper homeostasis. Copper absorption from dietary sources takes place in the gut through Ctr1 and DMT1, and endocytosis. At the enterocyte the Cu transporting ATPase ATP7A is in charge of delivering this copper into the secretory Cu proteins that will transport it to the liver. Copper is then transported in the bloodstream by ceruloplasmin and to the tissues through ATP7A. Alternatively, excess copper is excreted from the liver to the bile by means of ATP7B and into the gut (Adapted from Collins, 2017).

The traditional concern for Cu in fish has been more related to its potential toxicity (Clearwater, *et al*., 2002; Tang *et al*., 2013; Watanabe *et al*., 1997; Woody and O'Neal, 2012). In the context of marine aquaculture, the increase in the use of plant feed ingredients is altering the mineral profile of the commercial feeds. Contrary to the case of other minerals, such as Se and Zn, Cu is actually present in higher amounts in plant feed ingredients (Sanden *et al*., 2017), thus increasing the risk of a potential toxicity.

1.8b. 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; Clearwater *et al*., 2002). Several studies have focus on Cu requirements in the last decades, the following table focuses on those mainly orientated towards an understanding of the potential toxicity or requirements of dietary Cu (Table 1.4).

Table 1.4 Available literature on copper requirement studies in fish

Species	Scientific name	Requirement (mg Cu kg^{-1})	References
Atlantic salmon	*Salmo salar*	8.5–13.7 [1, 3]	Lorentzen *et al*., 1998
Blunt snout bream	*Megalobrama amblycephala*	11.8-18.0 [1, 2, 4]	Shao *et al*., 2012
Common carp	*Cyprinus carpio*	3.0 [1, 3]	Ogino and Yang, 1980
Channel catfish	*Ictalurus punctatus*	5.0 [4, 6]	Wilson and Gatlin, 1986
Crucian carp	*Carassius auratus gibelio*	6.5-9.4 [1, 2, 3, 4]	Shao *et al*., 2010

Table 1.4 *continued* Available literature on copper requirement studies in fish

Grass carp	*Ctenopharyngodon idella*	4.8 [1], 4.7 [2], 5.0 [5]	Tang *et al*., 2013
Hybrid tilapia	*O. niloticus X O. aureus*	4.0 [1, 2, 3, 5, 6]	Shiau and Ning, 2003
Large yellow croaker	*Larimichthys croceus*	3.4 [1], 5.3-5.9 [3], 7.1 [4]	Cao *et al*., 2014
Malabar grouper	*Epinephelus malabaricus*	4.0-6.0 [1, 2, 3, 4]	Lin *et al*., 2008b
		2.0-3.0 [1, 2, 3, 4]	Lin *et al*., 2010
Murrel	*Channa punctatus*	6.7-6.8 [1, 2, 4, 6]	Abdel-Hameid *et al*., 2017
Nile tilapia	*Oreochromis niloticus*	0.0-8.0*[as supplemented 1, 2, 5, 6]	Damasceno *et al*., 2016
Rainbow trout	*Oncorhynchus mykiss*	3.0 [3]	Ogino and Yang, 1980
Rockfish	*Sebastes schlegeli*	22.0*[present in basal diet 1, 2, 3, 6]	Gundogdu *et al*., 2009
		0.0*[from basal diet as supplemented 2, 6]	Kim and Kang, 2004
Russian sturgeon	*Acipenser gueldenstaedtii*	7.0-8.0 [1, 3, 4, 5]	Wang *et al*., 2016
		5.0 [CuMet/CuONano]	Wang *et al*., 2018b
		8.0 [$CuSO_4$ 1, 2, 3, 4, 5, 6]	
Tongue sole	*Cynoglossus semilaevis*	11.0-12.0 [1, 2, 4, 6]	Wang *et al*., 2015a
Yellow catfish	*Pelteobagrus fulvidraco*	3.2-4.6 [1, 2, 3]	Tan *et al*., 2011b

Criteria used to evaluate requirements: 1- Final weight/ weight gain, 2- Feed efficiency/ feed intake/ FCR, 3-Cu tissue retention, 4- Antioxidant activity markers (including TBARs, CuZnSOD, GPx, etc), 5- Ceruloplasmin activity or other Cu transporters, 6- Other markers.

1.8c. Deficiency and toxicity

Low Cu levels in the diet often produce a reduction in Cu tissue levels and lead to reduced feed efficiency and growth (Ogino and Yang, 1980; Tang *et al*., 2013), however, Cu deficiencies are rare.

The potential toxic effects of Cu on fish 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 (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 absorbed through 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 at daily Cu doses as low as 0.4–0.9 mg Cu kg^{-1} body $weight^{-1}$ (Murai *et al*., 1981). Actually, the same redox properties that allow Cu to eliminate free radicals enable it to promote reactions that lead to the production of reactive oxygen species, which can have damaging oxidative effects on cellular macromolecules. Cu may also manifest toxicity by displacing Zn from functionally essential protein domains (Minghetti *et al*., 2010).

1.8d. Sources

Cu content in feeds may vary considerably depending on the batch of ingredients utilised due to the presence of Cu containing contaminants (Lall, 2002). Dietary supplements of Cu are of importance and should be considered (Wang *et al*., 2015b). Copper sulphate ($CuSO_4 \cdot 5H_2O$) is a common source of Cu supplementation in fish feeds, however other sources for Cu include Cu chlorides, nanoparticles, Cu-methionine and other amino acid chelates (El Basuini *et al*., 2016; Shao *et al*., 2010; Shaw and Handy, 2006; Wang *et al*.,

2015b). Tribasic Cu chloride is a good source for gibel carp (Shao *et al*., 2010), while nanocopper is more effective for red sea bream (El Basuini *et al*., 2016) as well as for Russian sturgeon (*Acipenser gueldenstaedtii*; Wang *et al*., 2018b). On the other hand, chelated Cu is a more efficient source for Japanese sea bass (Wang *et al*., 2015b).

1.8e. Biomarkers

Several molecular markers can be affected by Cu, such as regulators of oxidative damage like Cu-Zn superoxide dismutase gene (*cuznsod*, Antony Jesu Prabhu *et al*., 2016; Lorentzen *et al*., 1998) or catalase gene (*cat*, Shao *et al*., 2012; Tang *et al*., 2013), and Cu transporters like copper transporter 1 gene (*ctp1*, Minghetti *et al*., 2008) and *atp7b* (Isani *et al*., 2011; Lanno *et al*., 1987). 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 (Carpenè *et al*., 1999) and the effects of toxic levels of Cu on metallothionein (Ghedira *et al*., 2010).

Cu deposition in liver and whole body have also been used in Cu requirement determination studies (Antony Jesu Prabhu *et al*., 2016; Lall, 2011; Lorentzen *et al*., 1998). In rockfish liver is a more important storage organ than other tissues, and the order of Cu accumulation was liver > intestine > kidney > gill >muscle (Kim and Kang, 2004). High levels of Cu supplementation may lead to hepatic alterations related to liver damage (Handy *et al*., 1999; Shaw and Handy, 2006).

OBJECTIVES

Within the current situation of the aquaculture industry, the tendency is to increase the use of plant ingredients to the detriment of unsustainable ingredients derived from fisheries. This shift brings with it a series of alterations in the mineral profile of the feed that are still to be fully understood. Gilthead sea bream mineral nutrition has been paid little attention despite being one of the flagships of European aquaculture. The hypothesis of this thesis was that the present reduction of FM and FO in commercial diets may affect the balance of dietary minerals and their optimum levels in diets for gilthead sea bream and that the type of mineral source supplemented may also affect dietary mineral utilization. Since the number of minerals to be studied for gilthead sea bream was very large, as a preliminary study that was part of a master thesis (Domínguez, *submitted a*), a complete mineral premix was fed to gilthead sea bream in 6 different concentrations in practical diets with high inclusion levels of plant ingredients, and a large variety of parameters specific for each mineral were determined. That study gave a good approximation of the optimum dietary levels for the different minerals and, particularly, clarified requirements for inorganic Zn. However, in that study, neither the optimum levels for other minerals with antioxidant functions such as Mn, Se or Cu were elucidated nor the importance of the different sources of minerals were studied. Therefore, the first part of the present thesis aimed to shed light on the use of different mineral sources, currently available for few antioxidant minerals (Mn, Se and Zn) and their combination in practical diets with high inclusion levels of plant ingredients, whereas the second part consisted of a series of studies with several levels of single minerals aimed towards better understanding the requirements of Mn, Se and Cu in gilthead sea bream juveniles fed practical diets.

More specifically, the objectives of this Thesis included:

1.- To understand the effect of different sources of minerals (Zn, Mn and Se) supplemented to practical diets with high levels of plant ingredients on gilthead sea bream growth performance and selected indicators of the nutritional status of those minerals these minerals. For that purpose a trial was conducted with a representation of available mineral sources namely, inorganic, (Mn and Zn oxides and NaSe), organic (Mn and Zn amino acid chelates and Se methionine), and inorganic encapsulated minerals.

2.- To better understand the specific effects of the different mineral sources for Mn and Zn, these minerals were tested in a trial with gilthead sea bream juveniles fed their inorganic sources as oxides, their organic sources as amino acid chelates, and combinations of these sources.

3.- To determine the optimum level of Mn supplementation for gilthead sea bream juveniles fed diets with high levels of plant ingredients.

4.- To define the effect of Se supplementation in practical diets for gilthead sea bream juveniles containing high levels of plant ingredients.

5.- To establish safe levels of Cu supplementation in diets for gilthead sea bream juveniles with high levels of plant ingredients.

CHAPTER 2: MATERIAL AND METHODS

2.1. PRODUCTIVE PARAMETERS

Growth, in terms of standard length (cm) and weight (g), was recorded in the different trials at different periods according to the experimental design, but always at the beginning and at the end of the trial by measuring and weighing all fish individually. Throughout the experiments, feed intake per replicate was recorded. At the end of the trials productive parameters were calculated including Specific Growth Rate (SGR), Feed Efficiency (FE), Feed Conversion Ratio (FCR), Weight Gain (WG) and Thermal Growth Coefficient (TGC), as well as survival rate using the following formulae:

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

$$\text{FE} = \frac{Final\ weight - Initial\ weight}{Total\ feed\ ingested}$$

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

$$\text{WG} = \left(\frac{Final\ weight - Initial\ weight}{Initial\ weight}\right) * 100$$

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

$$\text{Survival rate (\%)} = \frac{\text{nº final fish}}{\text{nº initial fish}} * 100$$

Where

W1: initial body weight (g)

W2: final body weight (g)

Temp: Temperature (ºC)

2.2. SAMPLING PROTOCOLS

Growth in terms of standard length (cm) and weight (g) was recorded by measuring and weighing all the fish. Previous to sampling, all fish were submitted to 24 h fasting. During samplings fish were caught and introduced into an anaesthetic tank containing clove oil (2ml/100l) (Guinama S.L.U., Valencia, Spain) to reduce stress and improve handling. Those fish that were meant to be returned to the tanks were recovered in a tank with abundant aeration and water flow until symptoms of recovery appeared (recuperation of verticality and sense of equilibrium, normal movement and response to external stimuli). Fish used for samples were sacrificed with excess clove oil and ice.

2.3. BIOCHEMICAL ANALYSES

At the beginning of the experiments 3 pools of fish were sampled to conduct biochemical analysis. At the final sampling, fish were sampled for biochemical analysis of tissues, as well as for fatty acid methyl esters (FAMES) when necessary. Samples were kept frozen at -80ºC until the analysis were conducted. Results for biochemical composition are expressed in terms of percentage dry matter (%DM) or wet weight.

2.3a. Crude protein

Proteins were calculated based on the total nitrogen composition of the samples by means of the technique described by Kjeldhal (AOAC, 2000). The method consists in the digestion of the samples with concentrated sulphuric acid at 400ºC in presence of a Cu catalyser, followed by distillation. The method is divided into three steps:

1) Digestion of the sample with concentrated sulphuric acid in presence of a Cu-Se catalyser in order to convert the nitrogen into ammonium sulphate.

2) Liberation of ammonia from the digested sample by addition of sodium hydroxide in excess and vapour distillation of this ammonia in boric acid 1%.
3) Determination of the liberated ammonia and titration with standard hydrochloric acid (0.1M).

Then, the following formula was applied to obtain the % protein in the sample and applying the correcting value of 6.25:

$$\% \text{ protein} = \frac{consumed\ HCl\ per\ sample\ (ml) - consumed\ HCl\ per\ white\ sample\ (ml)}{sample\ weight\ (mg)} * 100$$

2.3b. Crude lipids

The analytical method used to determine total crude lipids in the sample was based in that described by Folch *et al.* (1957), extracting the lipids by a mixture of chloroform-methanol (2:1) containing 0.01% (Butylated hydroxytoluene) BHT. The solvent is then evaporated by a stream of nitrogen; later on the remaining lipids are weighed and stored in a nitrogen atmosphere and dissolved in chloroform to avoid oxidation. The content of crude lipids is then calculated using the following formula:

$$\%\ lipids = \frac{\text{lipids (g)}}{\text{sample (g)}} * 100$$

2.3c. Ash content

Ash content is determined by incineration of the sample using a muffle furnace at 600ºC until constant weight (AOAC, 2000). The ash content is then calculated as:

$$\% \text{ ash} = \frac{final\ sample\ weight\ (g)}{initial\ sample\ weight\ (g)} * 100$$

2.3d. Moisture content

Moisture is calculated by drying the sample in an oven at 110ºC until constant weight (AOAC, 2000). The moisture content is then calculated by the formula:

$$\%\ moisture = \frac{\text{final sample weight (g)}}{\text{initial sample weight (g)}} * 100$$

2.3e. Fatty Acid Methyl Esters (FAMEs)

For fatty acids determination, total lipids are trans-esterified in sulfuric acid (1%) and methanol according to Christie (1982). FAMES are diluted in hexane and the separation, identification and quantification are carried out using gas chromatography (GC-14A, Shimadzu, Japan) as described by Izquierdo *et al*. (1992); results are expressed as g fatty acid/100g total fatty acids. In the heading "results" results for FAMES are expressed as PUFA (Polyunsaturated Fatty Acids) and LC-PUFA (Long Chain PUFA, which comprise those fatty acids with 20 carbons or more), as well as DHA (Docosahexaenoic acid 22:6(n-3)) and EPA (Eicosapentaenoic acid 20:5(n-3)).

2.3f. Thiobarbituric Acid-Reactive Substances (TBARs)

Thiobarbituric acid-reactive substances (TBARs) were measured from triplicate samples following Burk *et al*. (1980). Approximately 20–30mg of tissue were homogenised in 1.5ml of 20% trichloroacetic acid (w/v) containing 0.05 ml of 1% butylated hydroxytoluene in methanol. Then, 2.95 ml of freshly prepared 50mM-thiobarbituric acid solution were added before mixing and heating for 10min at 100ºC. After cooling, protein precipitates were removed by centrifugation at 2000G and the supernatant was read in a spectrophotometer (Evolution 300; Thermo Scientific, Spain) 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.

2.4. MINERAL COMPOSITION

With the aim of understanding how the different diets modulated the mineral composition of the fish, fish were taken at the beginning and end of the trials, and several tissues were evaluated, including whole body, liver and vertebrae. Samples were homogenised, lyophilized and stored at -80ºC prior to analysis.

The analysis consisted of a digestion of the samples in nitric acid and the posterior evaluation of the mineral content by means of an inductively coupled plasma mass spectrometry (ICP-MS).

2.4a. Digestion

Digestion takes place in a Milestone Microwave Support System (Milestone Srl, Bolonia, Italy). The samples are added distilled water, HNO_3 and H_2O_2 and submitted to a pressure of 50Bar. Microwaves are absorbed by the molecules/ions in the solution and put into rotary motion. The rotation leads to increase in the kinetic energy, which in turn gives rise in temperature. This results in the decomposition of the molecules/ions in the solution. The tests concluded on UW using a program that increases the temperature stepwise up to 260ºC. The program lasts 62 minutes: 37 minutes are used to perform the digestion of the samples (Table 2.1) and 25 minutes to cool the samples until they can be safely handled.

Table 2.1 Temperature program for sample digestion

Temperature program:
10 min increase to 80ºC
2 min at 80ºC
5 min increasing temperature up to 105ºC
5 min at 105ºC
10 min increase temp to 260ºC
5 min at 260ºC

2.4b. Mineral analysis

An inductively coupled plasma mass spectrometry (ICP-MS) "Thermo Scientific-iCAPQ" (Thermo Fisher Scientific Inc. Waltham, Massachussetts, USA) was used to determine mineral content. The conditions for the analysis are described in Table 2.2.

Table 2.2 Conditions for mineral analysis with ICP-MS

Parameter	Measure
Software	Qtegra y LIMS[1]
Nebulizer gas flow	0.9 l/min
Radio frequency	1200 W
Cool gas flow	13 l/min
Auxiliary gas flow	0.8 l/min
Helium gas collision cell flow	5 ml/min

[1] Qtegra y LIMS: developed by Thermo Fisher Scientific Inc. Waltham, Massachussetts, USA

2.5. VERTEBRAL MORPHOMETRY AND SKELETAL ANOMALIES

Radiographs were taken 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). Fish were radiographed in groups of ten individuals. Radiographs were treated digitally (Onis 2.4, DigitalCore, Co.Ltd, Tokyo, Japan). Additionally skeletal anomalies were classified according to Boglione *et al*. (2001).

2.6. HISTOLOGY

Fish were sampled for histological analysis at the end of the trials. 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. This process substitutes water and fat from the tissues, allowing the staining of the sample. Once the paraffin block is 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 (H&E) (Martoja and Martoja-Pearson, 1970) for optical evaluation. Once the preparations were ready they were subjected to 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 (Figures 4, 5, 6 and 7).

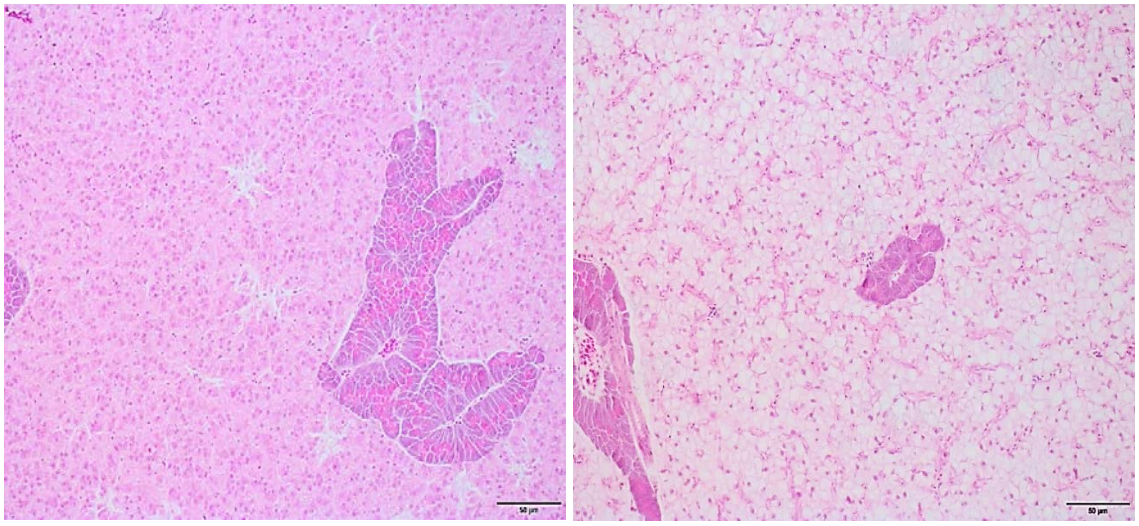

Figure 5. Microscopic view of liver (H&E) steatosis (20x). Left) Low steatosis. Right) High steatosis.

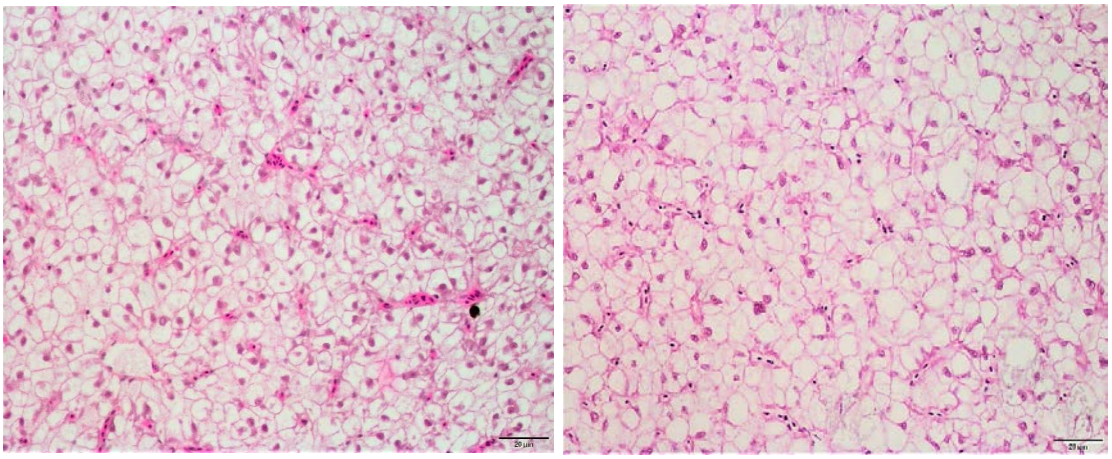

Figure 6. Microscopic view of liver (H&E) cell nucleus (40x). Left) Central nucleus. Right) Peripheral nucleus.

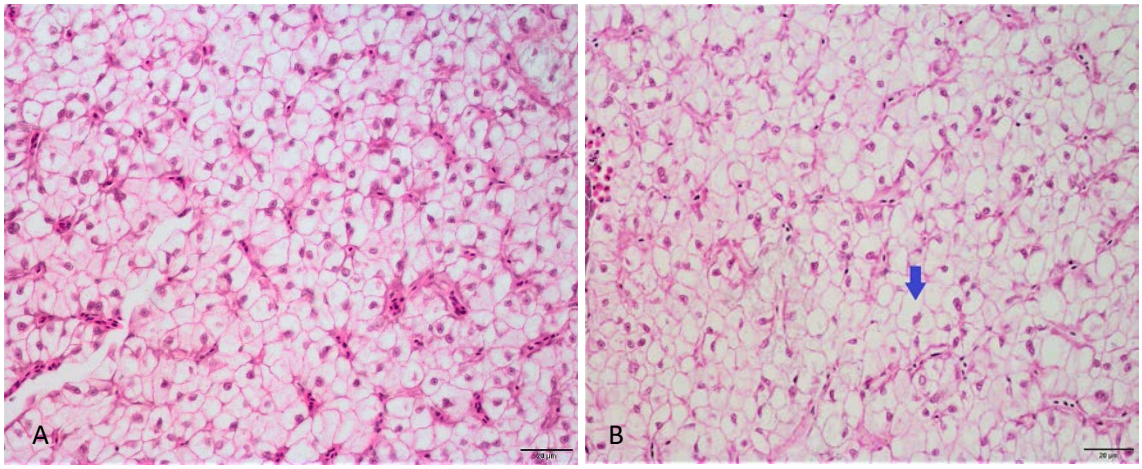

Figure 7. Microscopic view of liver (H&E) cell margin (40x). Left) well-preserved cell margins. Right) Broken cell margins.

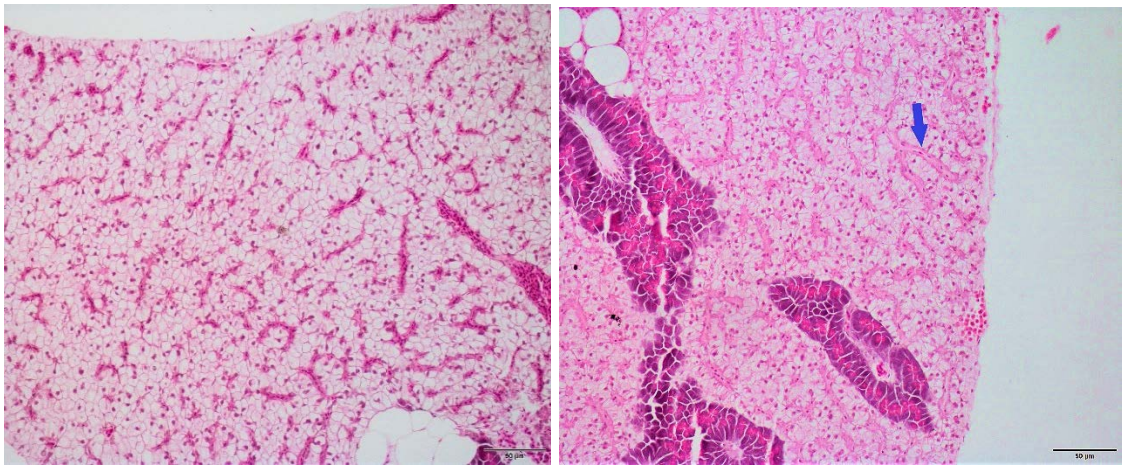

Figure 8. Microscopic view of liver (H&E) cell sinusoids (20x). Left) Sinusoids with abundant erythrocytes. Right) Sinusoids dilated with plasma and with a reduced number of erythrocytes.

2.7. GENE EXPRESSION

Five fish per tank were sampled at the end of the experiment for gene expression evaluation.

2.7a. Molecular cloning and gene expression analysis

Preparation of total RNA and first-strand (cDNA) synthesis

Total RNA was extracted from sea bream liver (100 mg) and posterior vertebrae (150 mg) using Maxwell®16 LEV simplyRNA Tissue kit (Promega, Italy), following the manufacturer's protocol. The quantity of the RNA was calculated at an absorbance of 260 nm. The integrity and relative quantity of RNA was assessed by electrophoresis. After extraction, 1 µg of total RNA from liver was reverse transcribed into cDNA in a final volume of 20 µl containing 1 µl of oligo dT16 primer (50 pmol) and 1 µl of 10 mM dNTPs, 4 µl of 5X reverse transcription buffer, 2 µl of 0.1 M DTT, 1 µl RNaseOUT (Invitrogen, Italy), and 1 µl of SuperScript® III Reverse Transcriptase (Thermo Fisher Scientific, Italy). After incubation at 50°C for 60 min, the reaction was stopped by heating at 70°C for 15 min.

2.7b. Real time RT-PCR quantification of gene expression

i) Generation of in vitro-transcribed mRNAs for each target gene

The number of transcript copies of genes *α-act* (Alpha-actin), *atp7b* (ATPase 7b), *bact* (Beta actin), *bmp2* (Bone morphogenetic protein 2), *cat* (Catalase), *cuznsod/sod* (Cu-Zn superoxide dismutase), *gr* (Glucocorticoid receptor), *gpx-1* (Glutathione peroxidase 1), *mnsod* (Mn superoxide dismutase), *oc* (osteocalcin) and *rpl-27a* (Ribosomal protein 27a), was quantified by using One Step Taqman real-time RT-PCR technique with the standard curve method. Standard curves were constructed using synthetic mRNAs with known number of copies for each gene. To obtain synthetic mRNAs, a forward and a reverse primer were designed based on the mRNA sequences of each gene and used to create templates for the in vitro transcription of mRNAs. The sequences of the primers used for the synthesis of synthetic mRNAs are reported in table 2.3.

Each forward primer was engineered to contain a T3 or T7 phage polymerase promoter gene sequence to its 5′ end and used together with the reverse primer in a conventional RT-PCR of total gilthead sea bream liver RNA. PCR products were then evaluated on a 2.5% agarose gel, cloned using pGEM®-T cloning vector system (Promega, Italy), and subsequently sequenced. *In vitro* transcriptions were performed using T3 or T7 RNA polymerase and other reagents supplied in the Promega RiboProbe in Vitro Transcription System kit, following the manufacturer's protocol.

The molecular weight (MW) of the *in vitro*-transcribed RNA for each target gene was calculated according to the following formula:

MW = [129 (no. of A bases) × 329.2) + 69 (no. of U bases) × 306.2) + 66 (no. of C bases) × 305.2) + 98 (no. of G bases) × 345.2)] + 159.

Spectrophotometry absorbance at 260 nm gave the concentration of each *in vitro*-transcribed RNAs. The concentration was used to calculate the number of moles [concentration (ng/µl)/MW], which was then multiplied for the Avogadro number to obtain the number of molecules/µl for each target gene mRNA.

ii) Generation of standard curves

The mRNAs produced by *in vitro* transcription were used as quantitative standards in the analysis of experimental samples. Defined amounts of mRNAs at 10-fold dilutions were run in real-time PCR using iTaq^{TM} Universal Probes One-Step Kit (Bio-Rad, Italy). Real time RT-PCR conditions were: 10 min at 50°C, and 3 min at 95°C, followed by 40 cycles consisting of 15 s at 95°C, and 1 min at 60°C. The Ct values obtained by real time PCR amplification were used to create standard curves for target genes.

iii) Quantification of target gene expression by one-step real-time RT-PCR

One hundred ng of total RNA extracted from the experimental samples were run in a One-Step quantitative real-time RT-PCR, in parallel to 10-fold-diluted defined amounts of standard mRNAs, under the same reaction conditions. Real-time PCR primers were designed using Primer Express (Life Technologies) and produced by Thermo Fisher Scientific (Italy) and Metabion International AG. The expression levels of *α-act* (Alpha-actin), *atp7b* (ATPase 7b), *bact* (Beta actin), *cat* (Catalase), *cuznsod/sod* (Cu-Zn superoxide dismutase), *gr* (Glucocorticoid receptor), *gpx-1* (Glutathione peroxidase 1), *mnsod* (Mn superoxide dismutase) and *rpl-27a* (Ribosomal protein 27a), were evaluated in liver, while *bmp2* (Bone morphogenetic protein 2) and *oc* (osteocalcin) were evaluated in vertebrae. RT-PCR reactions were run on a StepOne Real Time PCR System (Thermo Fisher Scientific, Italy). To reduce pipetting errors, master mixes were prepared to set up duplicate reactions (2×20 µL) for each sample. Primer sequences of each target gene are listed in Table 2.3.

iv) Sample quantification

Data from the TaqMan® PCR runs were collected with StepOne™ Software v 2.0. Ct (cycle threshold) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values were then used to create standard curves to serve as a basis for the calculation of the copies of mRNAs in total RNA.

Table 2.3 Nucleotide sequences of primers used in the studies

Gene	Symbol	Nucleotide Sequence
Alpha-actin	*α-act*	F: 5'-TCTGTCTGGATCGGAGGCTC-3'
		R: 5'-AAGCATTTGCGGTGGACG-3'
ATPase 7b	*atp7b*	F: 5'- CGCTGGCCTCGTGCTTCAACC-3'
		R: 5'-CGACGACCGCAGGCTTCTCATTT-3'
Beta actin	*bact*	F: 5'-TCTGTCTGGATCGGAGGCTC-3'
		R: 5'-AAGCATTTGCGGTGGACG-3'
Bone morphogenetic protein 2	*bmp2*	F: 5'-GTGGCTTCCATCGTATCAACATTTT-3'
		R: 5'-GCTCCCCGCCATGAGT-3'
Catalase	*cat*	F: 5'-ATGGTGTGGGACTTCTGGAG-3'
		R: 5'-AGTGGAACTTGCAGTAGAAAC-3'
Copper transporter 1	*ctr1*	F: 5'-CGGGTCTGCTCATCAACACCC-3'
		R: 5'-TGTGCGTCTCCATCAGCACCG-3'
Cu/Zn superoxide dismutase	*cuznsod*	F: 5'-TTGGAGACCTGGGCAACGTGA-3'
	/ sod	R: 5'-TCCTGCTTGCCTCCTTTTCCC-3'
Glucocorticoid receptor	*gr*	F: 5'-GGGCTGGATGGAAGAACGACA-3'
		R: 5'-ACACCGAAAGCACTGAGGAGG-3'
Glutathione peroxidase 1	*gpx-1*	F: 5'-GCTTTGAGCCAAAGATCCAG-3'
		R:5'-CTGACGGGACTCCAAATGAT-3'
Mn superoxide dismutase	*mnsod*	F: 5'-AGTGCCTCCTGATATTTCTCCTCTG-3'
		R: 5'-CCTGACCTGACCTACGACTATGG-3'
Osteocalcin	*oc*	F: 5'-AGCCCAAAGCAGGTAAGCAAG-3'
		R: 5'-TTTCATCACGCTACTCTACGG-3'
Ribosomal protein 27a	*rpl-27a*	F: 5'-ACAACTCACTGCCCCACCAT-3'
		R: 5'-CTTGCCTTTGCCCAGAACTT-3'

2.8. STATISTICAL ANALYSIS

All data were statistically analysed 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 and 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 $p<0.05$, and are expressed in the tables with superindexes. When data did not follow a normal distribution, logarithmic or arcsin transformations were carried out or non-parametric tests, such as Kruskal-Wallis, were used. Quadratic and linear regressions were used to establish a relation between levels of micronutrients and their effect on the different biomarkers.

CHAPTER 3: INORGANIC, ORGANIC, AND ENCAPSULATED MINERALS IN VEGETABLE MEALS BASED DIETS FOR *Sparus aurata* (LINNAEUS, 1758)

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3.1. ABSTRACT

Substituting fishmeal (FM) with vegetable meal (VM) can markedly affect the mineral composition of feeds, and may require additional mineral supplementation. Their bioavailability and optimal supplementation levels depend also on the form of delivery of minerals. The aim of the study was to determine the effect of different delivery forms of three major trace elements (Zn, Mn and Se) in a marine teleost. Gilthead sea bream juveniles of 22.5 g were fed a VM-based diet for 12 weeks that was either not supplemented with these minerals or supplemented with inorganic, organic, or encapsulated inorganic forms of minerals in triplicate and compared to a FM-based diet. Our results showed that mineral delivery form significantly affected the biochemical composition and morphology of posterior vertebrae. Supplementation of VM-based diets with inorganic forms of the target minerals significantly promoted growth, increased the vertebral weight and content of ash and Zn, enhanced bone mineralization and affected the vertebral shape. Conversely, encapsulation of inorganic minerals reduced fish growth and vertebral mineral content, whereas supplementation of organic minerals, enhanced bone osteogenesis by upregulating bone morphogenetic protein 2 (*bmp2)* gene and produced vertebrae with a larger length in relation to height. Furthermore, organic mineral forms of delivery downregulated the expression of oxidative stress related genes, such as Cu/Zn superoxide dismutase (*cuznsod*) and glutathione peroxidase 1 (*gpx-1*), suggesting thus that dietary minerals supplemented in the organic form could be reasonably considered more effective than the inorganic and encapsulated forms of supply.

3.2. INTRODUCTION

Gilthead sea bream (*Sparus aurata*) is one of the main marine finfish produced in the European aquaculture (APROMAR, 2015). Commercial feeds for gilthead sea bream have been traditionally based in fishmeal (FM) and fish oil (FO). These ingredients are limited resources with a tendency to decrease their production (Tacon and Metian, 2008; 2009). Vegetable meals (VM) and oils (VO) can partially replace FM and FO in gilthead sea bream diets (Benedito-Palos *et al*., 2007; Gómez-Requeni *et al*., 2004; Izquierdo *et al*., 2005; Montero *et al*., 2003; Robaina *et al*., 1995). However, this replacement may decrease antioxidant status (Saera-Vila *et al*., 2009). These issues may arise even when diets are formulated to satisfy the recommended nutrient requirements for essential fatty acids or amino acids (NRC, 2011). This suggests possible nutritional imbalances, which can be partly related to differences in the content of Zn, Mn, and Se between FM and VM (NRC, 2011; Hansen and Hemre, 2013), and can affect bone morphology and antioxidant status.

Zn is involved in bone formation and mineralization by activating osteoblastic cells and inhibiting osteoclastic bone resorption (Yamaguchi, 1998). Zn also forms part of several metalloenzymes that are involved in antioxidant defence such as Cu/Zn-SOD. In Jian carp the activity of Cu/Zn-SOD was increased with increasing dietary Zn levels (Feng *et al*., 2011). Low dietary Zn may cause slower growth rates in several fish species including Nile tilapia (Do Carmo e Sá *et al*., 2004), hybrid striped bass (Buentello *et al*., 2009), Jian carp (Tan *et al*., 2011a), grass carp (Liang *et al*., 2012), and Malabar grouper (Houng-Yung *et al*., 2014). In rainbow trout may also cause cataracts, skin, and fin erosion (Ogino and Yang, 1978), and dwarfism (Satoh *et al*., 1983). Studies focused on Zn requirement have been carried out on gilthead sea bream, using FM based diets (Serra *et al*., 1996). FMs contain high concentrations of Zn, whereas VMs are generally low in this mineral (NRC,

2011). For this reason, early studies in gilthead sea bream investigated the effects of increased Zn in diets in which FM was substituted by VM (Robaina *et al*., 1998).

Mn is a cofactor for metal-enzyme complexes, essential for the antioxidant defence through MnSOD. Gibel carp and yellow catfish fed a Mn-deficient diet showed reduced growth (Pan *et al*., 2008; Tan *et al*., 2012), whereas cataracts and dwarfism were described in rainbow trout and common and Gibel carps (Ogino and Yang, 1980; Pan *et al*., 2008; Satoh *et al*., 1983; Satoh *et al*., 1992; Yamamoto *et al*., 1983).

Similarly, Se plays an important role in reducing oxidative stress by being part of the selenoproteins such as glutathione peroxidase 1 *(gpx*-1), whose hepatic activity represent a robust and sensitive criterion to define Se deficiency (Fontagné-Dicharry *et al*., 2015; Pacitti *et al*., 2013). Se supplementation in diets for sea bass markedly reduced the occurrence of muscular dystrophy and oxidative risk, enhanced fish growth (Betancor *et al*., 2012) and adequate skeletal development (Saleh *et al*., 2014). Bone morphogenetic proteins (*bmp*) and osteocalcin (*oc*) are considered important indicators of bone development and mineralization and are positively related to Se inclusion (Saleh *et al*., 2014). *Bmp* are involved in a series of cascades that lead to osteoblast differentiation and osteogenesis in fish (Smith *et al*., 2006). Oc, instead, is an osteoblast-specific gene encoding a secreted protein, which represents the most abundant non collagenous protein of bone matrix (Sommer *et al*., 1996). This gene is generally inactivated during osteoblast proliferation, while it is abundantly transcribed during osteoblast differentiation. Osteocalcin is released by osteoblasts during bone formation and binds to the mineralized bone matrix (Hauschka and Wians, 1989).

Inorganic minerals may be more effectively absorbed if they are present in their chelated organic forms (Apines *et al*., 2004). Some authors have described an increase in Se

absorption in fish fed an organic source of Se (Paripatananont and Lovell, 1997). Similarly, several studies have shown that Zn and Mn are absorbed better when fish receive them in inorganic forms than organic or chelated to amino acids (Do Carmo e Sá *et al*., 2005; Watanabe *et al*., 1997). Other studies have reported higher bioavailability of these minerals when organic compounds are used instead of inorganic ones (Apines-Amar *et al*., 2004; Fountoulaki *et al*., 2010; Paripatananont and Lovell, 1995; Satoh *et al*., 2001). Minerals can also be delivered through encapsulation, thus reducing the interactions occurring with other minerals when they are supplied in excess. However, there is a lack of consistency in the repeatability of these results and important variations are found among different authors' data (Antony Jesu Prabhu *et al*., 2016).

Production of gilthead sea bream is constrained by the high incidence of skeletal anomalies, commonly reaching up to 100% (as cited in Boglione *et al*., 2001). Radiological studies serve as a useful method for classifying vertebral anomalies (Boglione *et al*., 2001; Witten *et al*., 2009). However, the relationship between vertebral mineral content and bone morphology has not been extensively studied with respect to experimental diets (Poirier Stewart *et al.*, 2014; Roy and Lall, 2003). Many radiological studies are based upon observations, and very few studies have actually used vertebral measurements to accurately describe vertebral morphology and anomalies (Fjelldal *et al*., 2007; Poirier Stewart *et al.*, 2014).

Accordingly, the objective of the present research was to further study the effects of Mn, Zn, and Se on bone development and oxidative stress markers by supplementing such minerals in different delivery forms in the gilthead sea bream diets for with low levels of FM and FO.

3.3. MATERIAL AND METHODS

The animal experiments described comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals and have been approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (REF: 007/2012 CEBA ULPGC).

3.3a. Diets

In order to determine the effect of supplementing low FM diets with Zn, Mn and Se by using different mineral delivery forms, five diets (manufactured by BioMar Tech-Centre, Brande, Denmark) were formulated (Table 3.1). Specifically, a low-FM, plant based diet (15% FM) was formulated without any supplementation of Zn, Mn and Se (negative control, C-). This basal diet, was then supplemented with the aforementioned target minerals in inorganic [DI, Zn oxide as ZnO (Zn 72%), Mn oxide as MnO_2 (Mn 60%), and sodium selenite], organic (DO, Se-methionine, Mn- and Zn-amino acids chelated) or inorganic encapsulated (DE). The encapsulated minerals were prepared according to Berthold *et al*., (1996) by SPAROS (Faro, Portugal) by precipitation-coacervation, in which chitosan was solved in a 2% (v/v) acetic acid solution and encapsulated particles were prepared by dropping the target mineral solution containing sodium selenite, Mn oxide and Zn oxide (Table 3.1). For comparison, a FM- and FO-based diet (FM) without supplementation of the target minerals was also included. For each of the supplemented experimental diets, a premix consisting of the target minerals in the different delivery forms (inorganic, organic and encapsulated) was prepared and added to the basal diet in order to ensure efficient mixing with the rest of the feed ingredients. Mineral composition of each diet was determined (Table 3.1). All diets were isoenergetic and isonitrogenous and were formulated to meet all known nutritional requirements for rainbow trout (NRC, 2011) including vitamins and minerals apart from the target ones (Table 3.1).

Table 3.1 Ingredients and analysed proximate composition of the experimental diets supplemented with different sources of target minerals (Zn, Mn and Se)

Ingredients (%)	**C-**	**DE**	**DO**	**DI**	**FM**
Fish meal[1]	15	15	15	15	63
Corn gluten	22	22	22	22	
Soya cake[2]	20	20	20	20	
Soya protein concentrate	10	10	10	10	
Wheat gluten	3.8	3.8	3.8	3.8	
Wheat	11.73	11.43	11.33	11.33	20.52
Fish oil[3]	7.5	7.5	7.5	7.5	8
Rapeseed oil[4]	7.5	7.5	7.5	7.5	8
Microingredients[5]	2.02	2.02	2.02	2.02	0.3
Premix vitamins and minerals[6]	0.45	0.45	0.45	0.45	0.45
Inorganic target minerals[7]				0.4	
Chelated (organic) target minerals[8]			0.4		
Encapsulated (chitosan) target minerals[9]		0.3			
Proximate composition (%)					
Moisture	8.1	8.2	8.1	8.1	7.0
Crude protein	44.2	43.7	43.5	42.5	45.0
Crude lipid	19.6	20.3	19.6	20.8	23.6
Ash	5.7	5.6	5.4	5.8	10.4
Mineral composition					
Zn (mg kg^{-1})	39	120	150	140	53
Mn (mg kg^{-1})	22	62	52	52	13
Se (mg kg^{-1})	0.55	0.89	1.20	0.90	1.70
Ca (%	0.95	0.98	0.86	1.00	2.20
P (%)	1.00	1.10	0.98	0.99	1.60
Ca:P	0.95	0.89	0.88	1.01	1.38

[1]South-American, Superprime.
[2]48 Hi Pro Solvent Extr.
[3]STD 18
[4]European, non-GM, double-low quality rapeseed oil
[5]Contains monocalcium phosphate, lysine, methionine and yttrium
[6]Contains vitamins and minerals to satisfy known nutritional requirements excluding the target minerals (Zn, Mn and Se) (DSM Nutritional Products, Basel, Switzerland).
[7]Zinc oxide, manganese oxide and sodium selenite (DSM Nutritional Products, Basel, Switzerland).
[8]Se Methionine, Zn, and Mn chelated to amino acids (DSM Nutritional Products, Basel, Switzerland).
[9]Encapsulated (chitosan) zinc oxide, manganese oxide and sodium selenite.

3.3b. Fish and experimental conditions

For the study, 1,725 gilthead sea bream (*Sparus aurata*) juveniles, obtained by natural spawning from our own broodstock (University of Las Palmas de Gran Canaria Las Palmas, Spain), were randomly distributed into fifteen 500-L circular fiberglass tanks at a density of 115 fish/ tank. Initial mean body weight and total length (TL) were 22.5 ± 1.5 g and 117 ± 4 mm (mean ± SD), respectively. Tanks in a flow-through system were supplied with filtered seawater at 22.8-24.3ºC and kept under a natural photoperiod (July-October) of approximately 12 h of light. Water-dissolved oxygen ranged between 6.5 and 6.9 ppm. Each diet was fed to triplicate groups until apparent satiation three times per day for 12 weeks. To monitor growth, individual fish were anesthetized with clove oil (Guinama S.L.U., Valencia, Spain) and weighed after 47 and 84 days of feeding. At the end of the study, 5 fish per tank were collected for whole body chemical composition; 20 for radiographic study; 8 fish per tank for vertebral axis weight, 6 fish per tank for vertebrae and liver gene expression studies, and 3 fish per tank for chemical and mineral composition of the vertebrae. Vertebrae gross chemical composition was determined from haemal vertebrae. Total vertebral weight was compared to total fish weight (vertebral weight/fish weight) to avoid the effect of weight differences.

3.3c. Biochemical analyses

Chemical composition of diets, and vertebrae was determined by 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 and at 550 ºC for vertebrae; protein content (N x 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. 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) at a private, certified laboratory (LDG, Barcelona, Spain), after submitting the sample to acid digestion.

3.3d. Vertebral morphometry

Radiographs were taken 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). Fish were radiographed in groups of ten. Radiographs were treated digitally (Onis 2.4, DigitalCore, Co.Ltd, Tokyo, Japan) and height, length and intervertebral spaces of eight different vertebrae were measured (vertebrae 3-6 and 13-16) (Fjelldal *et al.*, 2006). A series of measurements were used to describe vertebral morphometry, including vertebral height, vertebral length and vertebral surface area. Additionally, vertebral weight and length (mm) were studied in relation to total length (mm) of fish (vertebral weight/fish total length and vertebral length/fish total length) and the vertebral length and height relationship was calculated (vertebral length/vertebral height) to study vertebral shape. All these parameters were measured individually for each of the 20 fish per tank studied and served to define morphometric differences among fish fed the different diets.

3.3e. Gene expression

i. RNA extraction

Six fish were sampled from each tank and divided into two pools of three fish. Total RNA was extracted from 60 mg of liver and 150 mg of posterior vertebrae using TRI Reagent® Solution (Life Technologies, Carlsbad, CA, USA) and purified on RNeasy Mini Spin Columns (Qiagen, Hilden, Germany) following the manufacturer's instructions.

ii. 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 65ºC for 10 minutes and cooled in ice. Afterwards 1 µl of iScript reverse transcriptase and 4 µl of 5 x 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.

iii. Quantitative PCR

The nucleotide sequences of primers used in this study are reported in Table 3.2.

2 µl of diluted cDNA was used in real-time PCR for gene expression quantification using IQ^{TM} 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 20µl.

β-actin and ribosomal protein 27a (*rpl-27a*) were used as housekeeping genes to normalize the expression of oxidative stress genes (*gpx-1*, *sod*) in liver and of osteogenesis genes (*oc*, *bmp2*) in posterior vertebrae, respectively (Table 3.2). Real-time quantitative PCR was performed using 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 sec, followed by 40 cycles of 95°C for 15 sec, 58.1°C for 30 sec, and 72°C for 30 sec; 95°C for 1 min, and a final denaturation step from 58 to 95°C for 10 sec. The $2^{-\Delta\Delta Ct}$ method was applied to analyse the relative changes in gene expression.

Table 3.2 Sequences of primers used for gene expression analysis

Gene	Symbol	Nucleotide Sequence
Alpha-actin	*α-act*	F: 5'-TCTGTCTGGATCGGAGGCTC-3' R: 5'-AAGCATTTGCGGTGGACG-3'
Ribosomal protein 27a	*rpl-27a*	F: 5'-ACAACTCACTGCCCCACCAT-3' R: 5'-CTTGCCTTTGCCCAGAACTT-3'
Cu/Zn superoxide dismutase	*sod*	F: 5'-TTGGAGACCTGGGCAACGTGA-3' R: 5'-TCCTGCTTGCCTCCTTTTCCC-3'
Glutathione peroxidase 1	*gpx-1*	F: 5'-GCTTTGAGCCAAAGATCCAG-3' R:5'-CTGACGGGACTCCAAATGAT-3'
Bone morphogenetic protein 2	*bmp2*	F: 5'-GTGGCTTCCATCGTATCAACATTTT-3' R: 5'-GCTCCCCGCCATGAGT-3'
Osteocalcin	*oc*	F: 5'-AGCCCAAAGCAGGTAAGCAAG-3' R: 5'-TTTCATCACGCTACTCTACGG-3'

3.3f. Statistical analysis

All data were statistically analysed 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 the same or *Games-Howell* test whenever variances were different. Significant differences were considered for $p<0.05$. When data did not follow a normal distribution, logarithmic or arcsin transformation was carried out or non-parametric tests, such as *Kruskal-Wallis,* were used.

3.4. RESULTS

3.4a. Growth and productive parameters

Fish readily accepted experimental diets and no significant differences were found in feed intake between fish fed the different diets. From 47 days of feeding until the end of the trial, body weight was significantly lower in fish fed the VM-based diets, containing only 15% FM and 7.5% FO, than in fish fed the FM diet (Table 3.3). Whereas supplementation with Zn, Mn and Se in organic (DO) or encapsulated forms (DE) did not affect sea bream growth, addition of the same minerals in inorganic form (DI) significantly improved final body weight (Table 3.3). Moreover, in fish fed DI diet, the ratio between vertebral weight and total length ratio resulted significantly higher than in fish fed FM diet (Table 3.4).

Table 3.3 Body weight (g) of gilthead sea bream fed diets with different mineral sources at 0, 47, and 84 days

Body weight (g)	C-	DE	DO	DI	FM
0 days	22.5±0.8	22.6± 0.8	22.4±0.8	22.3±0.8	22.5±0.8
47 days	50.0±3.6^{a}	50.3±3.6^{a}	49.6±3.6^{a}	53.0±3.5^{b}	55.9±3.6^{c}
87 days	81.4±6.1^{a}	79.1±6.1^{a}	80.8±6.0^{a}	84.2±6.1^{b}	90.0±6.1^{c}

* Different letters in a row denote significant differences between groups fed different diets for a given feeding period (mean ± SD, n=3, $p<0.05$).

3.4b. Biochemical analyses

Fish belonging to different feeding groups did not differ in their whole body composition (Table 3.5). However, DI and DO diets significantly increased the ash content of posterior vertebrae in comparison to fish fed diet C- (Table 3.6). Feeding with diet DE did not increase ash content, but reduced protein content in the vertebrae compared to C-, DO and DI fed fish (Table 3.6).

Zn content in vertebra was increased by dietary supplementation of minerals regardless of mineral delivery form (Table 3.7). Mn and Se content in the vertebrae did not reflect the

amount of this mineral in the diet, which was lower in C- and FM diets, since no significant differences were found between the dietary fish groups (Table 3.7). Finally, among all fish groups, only sea bream fed the DI diet had a Ca and P vertebrae content significantly lower than fish receiving FM diet, although (Table 3.7). The dietary Ca and P levels did not differ significantly between the VM based diets (Table 3.1).

Table 3.4 Vertebral weight/fish total length, vertebral length/ fish total length, and vertebral length/ vertebral height of gilthead sea bream fed diets with different mineral sources for 12 weeks

Vertebral morphometry	C-	DE	DO	DI	FM
Vertebral weight/ fish total length (mg mm^{-1})	8.51 ± 2.10^{ab}	8.48 ± 2.29^{ab}	9.15 ± 1.76^{ab}	9.55 ± 2.03^{b}	8.24 ± 1.56^{a}
Vertebral length/ fish total length (mm $mm^{-1})^{-2}$	2.47 ± 0.11^{ab}	2.51 ± 0.07^{b}	2.49 ± 0.07^{ab}	2.47 ± 0.07^{a}	2.44 ± 0.09^{ab}
Vertebral length/ vertebral height (mm mm^{-1})	1.33 ± 0.07^{a}	1.39 ± 0.06^{c}	1.38 ± 0.07^{c}	1.36 ± 0.07^{b}	1.33 ± 0.08^{a}

Different letters in a row indicate significant differences in vertebral weight (mean ± SD, n=3, $p<0.05$) between experimental groups for the same time period.

Table 3.5 Initial and final whole body composition (% dry weight) of gilthead sea bream juveniles fed diets with different mineral sources for 12 weeks

	Initial	C-	DE	DO	DI	FM
Lipid	29.0±0.3	33.3±2.1	34.1±1.9	33.1±0.3	34.6±0.6	33.8±1.2
Ash	11.4±0.7	10.2±0.6	9.5±0.1	9.7±0.1	10.2±0.4	8.9±0.9
Protein	36.6±0.6	36.6±2.0	34.3±2.0	35.1±1.5	33.5±0.4	35.4±1.6
Moisture	67.7±0.9	66.6±0.4	66.2±0.4	66.2±0.6	66.4±0.6	65.6±0.3

Different letters in a row indicate significant differences in vertebral weight (mean ± SD, n=3, $p<0.05$) between experimental groups for the same time period.

Table 3.6 Initial and final composition (% dry weight) of posterior vertebrae of gilthead sea bream juveniles fed diets with different mineral sources for 12 weeks

	Initial	C-	DE	DO	DI	FM
Lipid	28.4±0.9	31.8±1.4	32.1± 3.4	32.5±2.0	33.3±0.9	32.0±1.7
Ash	33.0±1.8	32.3±1.8^{a}	32.3±1.5^{a}	34.2±1.1^{b}	34.9±0.9^{b}	33.5±1.5^{ab}
Protein	33.5±1.8	28.4±0.7^{b}	26.9±1.4^{a}	29.6±0.9^{b}	29.8±1.3^{b}	26.5±0.9^{a}
Moisture	47.8±1.3	43.6±2.2	44.2±1.2	40.3±7.3	44.3±0.3	43.3±1.0

Different letters in a row indicate significant differences (mean ± SD, n=3, p<0.05) between experimental groups for the same time period.

Table 3.7 Zn, Mn, Se, Ca, and P content in the posterior vertebrae of gilthead sea bream juveniles fed different mineral sources for 12 weeks

	Zn (mg kg^{-1})	Mn (mg kg^{-1})	Se (mg kg^{-1})	Ca (%)	P (%)	Ca:P
C-	31.4 ± 1.0^{a}	10.9 ± 0.2	0.15 ± 0.01	6.6 ± 0.5^{ab}	3.2 ± 0.2^{ab}	2.07
DE	34.7 ± 0.2^{b}	11.0 ± 0.3	0.15 ± 0.02	6.0 ± 0.5^{ab}	2.9 ± 0.3^{ab}	2.06
DO	34.9 ± 0.5^{b}	11.1 ± 0.5	0.17 ± 0.03	6.3 ± 0.6^{ab}	3.0 ± 0.3^{ab}	2.07
DI	35.2 ± 1.2^{b}	11.2 ± 0.2	0.18 ± 0.03	5.4 ± 0.9^{a}	2.7 ± 0.4^{a}	2.03
FM	33.4 ± 1.4^{ab}	10.7 ± 0.7	0.17 ± 0.03	7.0 ± 0.2^{b}	3.4 ± 0.2^{b}	2.04

Different letters in a row indicate significant differences (mean ± SD, n=3, p<0.05) between experimental groups for the same time period.

3.4c. Vertebral morphometry

From our previous studies on vertebral morphology, the posterior vertebra V13 resulted to be more affected by the diet than anterior vertebrae, and therefore it was used, in this study, to calculate vertebral morphometric parameters. Among the different parameters measured, the ratios between the vertebral length and fish total length and between the vertebral length and vertebral height were significantly affected by dietary treatments (Table 3.4). In general, inclusion of minerals to a VM-diet did not increase the vertebral length/fish total length ratio in comparison to fish fed diets C- and FM (Table 3.4). This value was significantly higher in fish fed DE diet compared to those receiving diet DI. Conversely, the shape of the vertebrae was significantly affected by mineral dietary inclusion. Specifically, compared to C- and FM groups, the vertebral length/vertebral height ratio was significantly increased by including encapsulated (DE) and organic (DO) target minerals,

and to a lesser extent by inorganic minerals (DI) (Table 3.4). Only fish fed DI diet showed, instead, a vertebral weight/fish total length value higher than FM group.

3.4d. Gene expression

Fish fed diet C- showed the lowest *oc* gene expression in vertebrae, being significantly lower than in fish fed FM diet (Table 3.8). Mineral supplementation to the VM based diets, improved the transcript levels of *oc*, but the high variation in the data and the small number of samples (n=6) did not allow to show significant differences. Similarly, expression of *bmp2* gene was the lowest in fish fed diet C- and it was increased by adding minerals, being significantly higher in fish fed organic minerals (Table 3.8). In liver *gpx-1* gene expression tended to be lower, although not significantly, in fish fed organic minerals (DO) as well as FM control diet. Conversely, fish fed encapsulated minerals (DE) showed a significant ($p < 0.05$) upregulation of *sod* gene, whilst those fed C- and FM diets presented the lowest expression. Consequently, fish receiving the diet supplemented with organic or inorganic form of minerals showed intermediate expression level of *sod* (Table 3.9).

Table 3.8 Expression level of osteocalcin (oc) and bone morphogenetic protein 2 gene (bmp2) in vertebrae of gilthead sea bream juveniles fed diets with different mineral sources for 12 weeks

Vertebral ossification related genes	C-	DE	DO	DI	FM
oc	1.00 ± 0.07^{a}	1.94 ± 0.95^{ab}	2.04 ± 0.47^{ab}	2.25 ± 0.61^{ab}	1.59 ± 0.11^{b}
bmp2	1.10 ± 0.58^{a}	3.31 ± 1.93^{ab}	7.75 ± 1.00^{b}	2.56 ± 0.49^{ab}	4.07 ± 1.38^{ab}

Different letters in a row indicate significant differences in gene expression (mean ± SD, n=6, $p<0.05$) between experimental groups for the same time period.

Table 3.9 Expression level for oxidative stress related genes (gpx-1 and sod) in liver of gilthead sea bream juveniles fed diets with different mineral sources for 12 weeks

Oxidative stress related genes	C-	DE	DO	DI	FM
gpx-1	1.01 ± 0.20	1.41 ± 0.59	0.85 ± 0.17	1.38 ± 0.20	0.86 ± 0.15
sod	1.00 ± 0.06^{c}	4.26 ± 0.14^{a}	1.91 ± 0.65^{bc}	2.85 ± 0.78^{b}	1.35 ± 0.38^{c}

* Different letters in a row indicate significant differences in gene expression (mean ± SD, n=6, $p<0.05$) between experimental groups for the same time period.

3.5. DISCUSSION

The dietary content of trace elements can affect fish performance and other biomarkers (Antony Jesu Prabhu *et al*., 2016; NRC, 2011). In the present study, the FM based diet was lower in Zn and Mn but higher in Se in comparison to the supplemented diets, whereas the VM-based diet that was without any mineral supplementation had lower content of all three target minerals. Dietary levels of Zn, Mn and Se in the non-supplemented diet (C-) (39, 22 and 0.55 mg kg^{-1}, respectively) were lower than the requirements described for marine fish species. For instance, Zn requirements for growth have been reported to be at least 61 mg kg^{-1} for sea bream (Carpenè *et al*., 1999) and 60.2 mg kg^{-1} for turbot (Ma *et al*., 2014), whereas Mn requirements are about 25 mg kg^{-1} for cobia (Liu *et al*., 2013). Se requirements are about 0.8 mg kg^{-1} for cobia (Liu *et al*., 2010), 1.6 mg kg^{-1} for malabar grouper (Lin and Shiau, 2005) and 1.6 mg kg^{-1} for juvenile largemouth bass (*Micropterus salmoide*; Zhu *et al*., 2012). The dietary content of three trace elements in our supplemented diets (DI, DO and DE) was instead higher than the requirements described for marine fish species, but, anyway, below to levels usually considered toxic.

In the present study, fish fed a VM-diet without supplementation of Zn, Mn and Se showed either reduced growth, vertebral length and/or vertebral mineralization of posterior vertebrae, in conjunction with low ash content and the lowest expression of *oc* and *bmp2*, which are biomarkers of bone differentiation and mineralization in gilthead sea bream (Saleh

et al., 2014). This could indicate a lack of Zn, Mn or Se, since a deficiency in these minerals has been related to reduced growth, dwarfism and bone malformation (Le *et al*., 2014; Watanabe *et al*., 1997). Diet containing inorganic trace elements significantly promoted growth in juvenile sea bream in comparison to the other diets. Dietary inorganic Zn has been found to improve growth in other marine and freshwater species, such as malabar grouper (Houng-Yung *et al*., 2014), yellow catfish (Luo *et al*., 2011), Jian carp (Tan *et al*., 2011a), and hybrid tilapia (Zhao *et al*., 2011). Nevertheless, dietary inorganic Mn could have also contributed to improve growth. Indeed, the increase in Mn has been shown to promote growth in gibel carp (Pan *et al*., 2008), cobia (Liu *et al*., 2013), and yellow catfish (Tan *et al*., 2012). In particular, in this study, inorganic Zn and Mn were supplemented in the form of oxides (ZnO and MnO_2, respectively) and Se was added as sodium selenite (Na_2SeO_3). The latter has been used in several previous trials and is considered an effective inorganic source of Se to fish (Antony Jesu Prabhu *et al.*, 2016). In most previous studies in fish, the inorganic forms of Zn and Mn that are commonly used for dietary supplementation were sulphates ($ZnSO_4$ and $MnSO_4$, respectively). In general, sulphates have been shown to have higher bioavailability than oxides in fish (Antony Jesu Prabhu *et al*., 2016; NRC, 2011) and other animals (EFSA Journal, 2012). However, dietary supplementation with ZnO showed no difference to $ZnSO_4$ on growth performance, feed utilization and bone Zn concentration and retention in tilapia (Do Carmo e Sa *et al*., 2005). Moreover, in this species, both Zn inorganic forms (ZnO and $ZnSO_4$) showed better results as compared to the amino acid chelated form. In sea bass, dietary supplementation with ZnO showed no significant difference to organic Zn in growth and feed utilization but had lower tissue bioavailability in skin and liver in comparison to the respective concentration of the organic Zn (Fountoulaki *et al*., 2010). Conversely, in carp, dietary Mn showed higher availability in the sulphate and chloride forms than in the oxide and carbonate ones (Satoh *et al*., 1987). To our knowledge, this is the first study to evaluate the ZnO and MnO_2 as mineral sources in sea bream diets in

a comparative way to organic and encapsulated forms. In line with previous evidences, our findings indicate that the efficiency of different inorganic mineral sources could be species dependent. Nevertheless, in this case, the positive results obtained by the inorganic forms used (i.e, oxides and salts) are promising mineral sources to be used in the supplementation of feeds for sea bream.

Mineral content in the vertebrae has been suggested as the main criterion for estimation of nutritional requirements for Zn and Mn, since fish mainly store these elements in the vertebrae (Antony Jesu Prabhu *et al*., 2016). The significantly lower Zn content in vertebrae of gilthead sea bream fed the diet non-supplemented with the target minerals might indicate a mineral deficiency; therefore, it means that gilthead sea bream juveniles have a Zn requirement higher than the 39 mg kg^{-1} ensured by the not supplemented diet, as previously recommended (Serra *et al*., 1996). Although Se concentration in cobia vertebrae increased with increasing dietary Se (Liu *et al*., 2010), Mn and Se content in the vertebrae of our fish did not vary significantly with mineral supplementation though. Lower vertebral Ca and P were found in fish fed inorganic minerals, whereas in the same fish group, vertebral weight and ash, protein and Zn content, increased. Zn has been found to stimulate bone formation through proliferation of osteoblastic cells (Yamaguchi, 1998), however rats fed with excessive levels of Zn (>2500 mg kg^{-1}) showed reduced bone Ca and P in the study of Stewart and Magee (1964). Detailed data of Zn requirements in sea bream is needed; therefore, further dose-response studies are being conducted by our group to understand the effect of organic and inorganic Zn in fish diets with high plant protein inclusion.

Feeding inorganic minerals enhanced the expression of *oc* that is a molecular marker of bone mineralization, and affected vertebral morphology, by increasing their length in relation to height as compared to the group fed with non-supplemented diet. The good correlation between dietary Zn levels and *oc* expression is in agreement with the promotion of bone

mineralization by this mineral. In rainbow trout, Zn supplementation at 60 mg kg^{-1} levels caused a statistically significant increase of serum alkaline phosphatase (ALP) activity and significantly decreased oxidative stress (Kucukbay *et al*., 2006). Serum ALP is of particular interest since an increase of its activity is usually associated to osteoblast hyperactivity and bone remodelling and, in human, ALP expression is one of the most frequently used markers of the osteoblast differentiation process (Huang *et al.*, 2013)

The response of oxidative stress related genes *gpx-1* and *sod* to different dietary mineral delivery forms was characterized by a decrease of their expression associated with mineral supplementation in organic delivery forms (DO). In particular, the supplementation of VM-based diet with organic minerals tended to downregulate the expression of *gpx-1* and significantly decreased *sod* transcription levels in liver of gilthead sea bream. Our results are in agreement with the downregulation of *gpx-1* gene expression found in gilthead sea bream larvae fed increased dietary organic Se levels, (Saleh *et al*., 2014). Se plays an important role in fish antioxidant defences being a cofactor of the antioxidant enzyme GPx (Felton *et al*., 1996). In sea bass larvae, an increase in dietary organic Se downregulated both *gpx-1* and *sod* with a consequent reduction of free radicals production (Betancor *et al*., 2012). *Sod* expression was reduced by dietary Se supplementation also in *Brycon cephalus* exposed to an oxidative stress-producing agent (Monteiro *et al*., 2009). However, if enzymatic activity is considered, the effect of Se is the opposite. Indeed, previous studies have reported that dietary supplementation of Se enhanced the antioxidant enzyme capacity in common carp (Elia *et al*., 2011), rainbow trout (Küçükbay *et al*., 2009), cod (*Gadus morhua*, Penglase *et al*., 2010) and yellowtail kingfish (Le and Fotedar, 2016). Based on *gpx-1* and *sod* expression data obtained in our study, organic Se seemed to be the most efficient delivery form. This assumption is in agreement with several evidences suggesting that organic Se at higher

concentrations is more bioavailable and tolerated than inorganic Se (Küçükbay *et al*., 2009; Paripatananont and Lovell, 1997; Rider *et al*., 2009, 2010; Thiry *et al*., 2012; Wang *et al*., 2007).

However, this was not entirely true for Zn and Mn. Indeed, gilthead sea bream fed minerals as inorganic oxides showed ash, Zn, and Mn vertebral content comparable to fish fed with organic minerals, thus denoting their high availability. In line with our result, in rainbow trout and hybrid striped bass, proteinate or amino acid chelated Zn did not increase Zn deposition in bone in comparison to inorganic form (Zn sulfate) (Rider *et al*., 2010; Savolainen and Gatlin, 2010). Nevertheless, in other fish species, higher absorption and retention of Zn and Mn were found when the minerals were supplemented in the organic form (chelated to amino acids or Zn propionate) than in the inorganic form (sulfate or oxide forms) (Apines *et al*., 2001; Hahn and Baker, 1993; Paripatananont and Lowell, 1997; Satoh *et al*., 2001; Spears, 1989; Wedekind *et al*., 1992). Therefore, it is important to carefully consider in which form minerals are supplemented: in species in which supplementation in organic form may increase mineral availability, it is necessary to reduce their inclusion levels, thus avoiding a possible negative effect due to excess of accumulation. For instance, daily doses of 9-12 mg Zn kg^{-1} body weight d^{-1} resulted to be toxic in carp, Nile tilapia, and guppy (*Poecilia reticulate*, Clearwater *et al.*, 2002). Conversely, dietary concentrations of Zn > 900 mg kg^{-1} dry diet were relatively nontoxic to several fish species (Clearwater *et al*., 2002). Further studies are being conducted to better understand the effects of inorganic and organic Zn and Mn on growth of sea bream.

Integration of VM-based diets with encapsulated minerals did not improve fish growth and led to a higher expression of *sod* genes in liver, suggesting an increase in the oxidative risk. In addition, Zn, Mn, and Se levels in vertebrae tended to be lower in fish fed encapsulated minerals, regardless of their dietary levels. The low protein content in fish fed encapsulated

minerals (similar to those fed the FM-based diet) could also be related to a lower Zn availability in its encapsulated form since this mineral has been found to increase the protein component of bone and to promote bone growth via insulin like growth factor 1 action (Ma and Yamaguchi, 2001a,b).

In conclusion, the results of this study showed that supplementation of inorganic Zn is required in VM-based diets to promote growth in gilthead sea bream. Organic minerals, particularly Se, seemed more effective in reducing oxidative risk, whereas encapsulated delivery forms reduced the ash content of the vertebrae, or in other words, mineral deposition, thus negatively affecting fish growth.

CHAPTER 4: EFFECTS OF ZINC AND MANGANESE SOURCES ON GILTHEAD SEA BREAM (*Sparus aurata*) FINGERLINGS

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4.1. ABSTRACT

The use of vegetable ingredients may alter the mineral balance of feeds for gilthead sea bream due to the differences in mineral composition between marine and vegetable ingredients. Whereas sea bream fed low levels of Zn and Mn may present reduced growth and performance, as well as alterations in mineral deposition and oxidative stress resistance, information on the type of sources of these two minerals is almost inexistent. In the present study, VM/VO based diets were formulated including the target minerals, Zn and Mn in two different delivery forms, oxides and chelated to amino acids. Gilthead sea bream of 8.2g were fed one of five different diets containing either non mineral supplementation, Zn and Mn oxides, amino acid chelates and their combinations. During the experimental period, fish fed Zn oxide presented higher growth, a higher deposition of protein and lower lipid contents in whole body, whereas there was an increase in whole body TBARs and up-regulation of *cuznsod* in fish fed diets supplemented with amino acids chelated Zn.

4.2. INTRODUCTION

The nutritional composition of feeds based on marine ingredients differ significantly to those based upon vegetable ingredients, and can cause negative effects such as increased lipid deposition in the liver (Robaina *et al.*, 1995) and reduced antioxidant status (Saera-Vila *et al.*, 2009). The use of vegetable ingredients as an alternative to fish derivatives may alter the mineral profile of feeds for gilthead sea bream (*Sparus aurata*) due to the differences in mineral composition between marine and vegetable ingredients. Knowledge about marine fish species regarding mineral requirements is scarce (Hamre *et al.*, 2013). Furthermore, currently there are no specific recommendations for Zn and Mn supplementation on gilthead sea bream diets. On the other hand, information

regarding Zn and Mn sources for sea bream fingerlings is very scarce (Dominguez *et al.*, 2017).

Zn is an essential trace element that takes part in up to 20 metalloenzymes implicated in lipid, carbohydrate and protein metabolism. Besides, Zn plays an important role in regulating oxidative stress and immunity, and intervenes in reproductive processes. Some of the indicators frequently used to establish Zn requirements comprises Zn contents in several tissues including whole body and vertebrae, and *cuznsod* activity or gene expression (Apines-Amar *et al.*, 2004; Gatlin and Wilson, 1983; Paripatananont and Lovell, 1995). Several forms are commonly used as Zn supplements in aquafeeds including oxides and amino acid chelates. The use of zinc oxide as an additive is considered by the European Food Safety Authority (EFSA) as a safe source of zinc for all animal species, with no concerns for consumer safety, and not expected to pose an appreciable risk to the environment (EFSA, 2012). However, Zn may be more effectively absorbed if it is chelated to amino acids (Apines-Amar *et al.*, 2004).

Mn is a transition metal that acts as a cofactor for essential metalloenzymes and contributes to prevent and reduce the negative effects of free radicals by forming part of Mn superoxide dismutase (MnSOD). *mnsod* activity and gene expression, as well as Mn contents in vertebrae, heart and whole body content, are used as indicators of Mn deficiency (Knox *et al.*, 1981; Maage *et al.*, 2000).

In gilthead sea bream, the combined supplementation of Zn, Mn and Se as inorganic (MnO_2, ZnO, NaSe) forms significantly increases growth, in comparison to supplementation of amino acid chelated organic forms or inorganic encapsulated forms (Dominguez *et al.*, 2017). However, supplementation of organic forms down-regulated the expression of oxidative stress related genes, such as *cuznsod* and glutathione

peroxidase 1 (*gpx-1*), suggesting a better utilization of dietary minerals. On the contrary, in gilthead sea bream larvae the combined inclusion of these three minerals as peptide chelated organic forms improved growth in comparison to inorganic ($MnSO_4$, $ZnSO_4$ and NaSe) forms (Izquierdo *et al.*, 2016), whereas expression of antioxidant enzymes genes was not affected (Terova *et al.*, 2018). Nevertheless, Se forms part of the potent antioxidant enzyme glutathione peroxidase and its supplementation can have direct effects on its expression in sea bream (Saleh *et al.*, 2014), as well as on sod expression in European seabass (*Dicentrarchus labrax*, Betancor *et al.*, 2012), which may interfere with the effects of Zn and Mn supplementation in our previous trials (Dominguez *et al.*, 2017; Izquierdo *et al.*, 2016). Therefore, the effects of Zn and Mn supplementation must be considered independently from those caused by Se supplementation.

The present study aimed to further clarify the effect of different combinations of organic (amino acid chelated) and inorganic (oxide) forms of Zn and Mn in diets for gilthead sea bream juveniles on productive, biochemical, genetic and morphological parameters.

4.3. MATERIAL AND METHODS

The animal experiments described comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals and have been approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (REF: 007/2012 CEBA ULPGC).

4.3a. Diets

In order to determine the effect of supplementing low FM diets with zinc (Zn) and manganese (Mn) by using different mineral sources, five experimental diets (manufactured by BioMar Tech-Centre, Brande, Denmark) were formulated (Table 4.1).

Specifically, a low-FM, plant based diet (15% FM) was formulated to devoid any added Zn and Mn (C-: negative control). This basal diet, was then supplemented with the aforementioned target minerals as oxides (ZnOx MnOx), amino acid chelates (ZnAA MnAA), or a combination of both sources (ZnAA MnOx: Zn-amino acids chelated and manganese oxide; ZnOx MnAA: zinc oxide and Mn-amino acids chelated) (Table 4.1). For each of the supplemented experimental diets, a premix consisting of the target minerals in the different delivery forms was prepared and added to the basal diet in order to ensure efficient mixing with the rest of the feed ingredients. All diets were isoenergetic and isonitrogenous and were formulated to meet all known nutritional requirements for sea bream juveniles including vitamins and minerals apart from the target ones (Table 4.1).

4.3b. Fish and experimental conditions

For the study, 3600 gilthead sea bream (*Sparus aurata*, Linnaeus 1758) juveniles obtained from a natural spawning from the GIA broodstock (Universidad de Las Palmas de Gran Canaria Las Palmas, Spain) were randomly distributed into fifteen 500-L circular fiberglass tanks at a density of 240 fish/tank. Initial mean body weight was 8.2 ± 0.4 g (mean ± s.d.). Tanks were supplied with filtered seawater and kept under a natural photoperiod (July-October) of approximately 12 h of light. Water temperature and oxygen were constantly measured by oxygen and temperature controllers and software (INNOVAQUA, Seville, Spain). Fish were manually fed until apparent satiation 3 times per day for 12 weeks. Each experimental diet was tested in triplicate. To monitor growth, individual fish were anesthetized with clove oil and weighed after 21, 42, 63 and 84 days of feeding. At the end of the study, 4 fish per tank for histological analysis of liver; 6 fish per tank for liver gene expression studies, and 10 fish per tank for proximate and mineral composition of the whole body and mineral composition of vertebrae.

Table 4.1 Ingredients and analysed composition of the experimental diets supplemented with different sources of Zn and Mn

COMPOSITION (%)	C-	ZnOx MnOx	ZnAA MnAA	ZnAA MnOx	ZnOx MnAA
Fish Meal SA Superprime 68%	15	15	15	15	15
Soya HP48, Non Gmo	20	20	20	20	20
Soya SPC >60%, non gmo	10	10	10	10	10
Wheat Gluten	4.8	4.8	4.8	4.8	4.8
Maize Gluten	22	22	22	22	22
Wheat	10.7	10.5	10.5	10.5	10.5
Fish Oil SA	7.5	7.5	7.5	7.5	7.5
Rapeseed Oil	7.5	7.5	7.5	7.5	7.5
Mono-calcium Phosphate (MCP)	1.41	1.41	1.41	1.41	1.41
L-Lysine	0.57	0.57	0.57	0.57	0.57
DL-Methionine	0.08	0.08	0.08	0.08	0.08
Yttrium	0.03	0.03	0.03	0.03	0.03
ARRAINA WP2 - Basal Premix	0.41	0.41	0.41	0.41	0.41
Premix ZnOx/MnOx		0.2			
Premix ZnAA/MnAA			0.2		
Premix ZnAA/MnOx				0.2	
Premix ZnOx/MnAA					0.2
Total	100	100	100	100	100

Table 4.1 *continued.* Ingredients and analysed composition of the experimental diets supplemented with different sources of Zn and Mn

BIOCHEMICAL COMPOSITION					
ENERGY - crude (MJ/kg)	21.51	21.51	21.51	21.51	21.51
MOISTURE (%)	9.35	9.35	9.35	9.35	9.35
PROTEIN - crude (%)	44.97	44.97	44.97	44.97	44.97
FAT - crude (%)	18.69	18.69	18.69	18.69	18.69
ASH (%)	6.68	6.68	6.68	6.68	6.68
STARCH - crude (%)	9.63	9.63	9.63	9.63	9.63
PHOSPHORUS - total (%)	1.02	1.02	1.02	1.02	1.02
NFE (%)	20.41	20.41	20.41	20.41	20.41
NSP - Crude (%)	10.78	10.78	10.78	10.78	10.78
EPA (C20:5 n-3)	13.97	13.97	13.97	13.97	13.97
DHA (C22:6n3)	10.13	10.13	10.13	10.13	10.13
EPA+DHA	24.1	24.1	24.1	24.1	24.1

Mineral composition (mg kg^{-1}) C- Zn: 49, Mn: 30; ZnOx/MnOx Zn: 140, Mn: 61; ZnAA/MnAA Zn:155, Mn: 70; ZnAA/MnOx Zn: 137, Mn: 59; ZnOx/MnAA Zn: 137, Mn: 66.

4.3c. Biochemical analyses

Biochemical analysis composition of diets, and whole fish was determined by 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 600ºC 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. 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).

Thiobarbituric acid-reactive substances (TBARS) were measured from triplicate samples following Burk *et al.* (1980). Approximately 20–30mg of tissue were homogenised in 1.5ml of 20% TCA (w/v) containing 0.05 ml of 1% butylated hydroxytoluene in methanol. Then, 2.95 ml of freshly prepared 50mM-thiobarbituric acid solution were added before mixing and heating for 10min at 100ºC. After cooling, protein precipitates were removed by centrifugation at 2000g 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-malonaldehyde (MDA), expressed as mmol MDA/g tissue was calculated using an extinction coefficient of 0.156 cm/mM.

4.3d. Gene expression

i. 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.

ii. 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.

iii. Quantitative PCR

The nucleotide sequences of primers used in this study are reported in Table 4.2. 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. Ribosomal protein 27a (*rpl-27a*) was used as housekeeping genes to normalize the expression of oxidative stress genes (*cuznsod* and *mnsod*) in liver (Table 4.2). 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 $2-\Delta\Delta Ct$ method was applied to analyse the relative changes in gene expression.

Table 4.2 Sequences of primers used for gene expression analysis

Full gene name	Gene	Nucleotide Sequence
Ribosomal protein 27a	*rpl-27a*	F: 5'-ACAACTCACTGCCCCACCAT-3' R: 5'-CTTGCCTTTGCCCAGAACTT-3'
Cu/Zn superoxide dismutase	*cuznsod*	F: 5'-TTGGAGACCTGGGCAACGTGA-3' R: 5'-TCCTGCTTGCCTCCTTTTCCC-3'
Mn superoxide dismutase	*mnsod*	F: 5'-AGTGCCTCCTGATATTTCTCCTCTG-3' R: 5'-CCTGACCTGACCTACGACTATGG-3'

4.3e. Histology

Four fish per tank were sampled for histological analysis of the 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 steatosis, and analysed by pair evaluators in a 0-3 scale, where 0 was absence of steatosis and 3 steatosis present in most of the liver.

4.3f. Statistical analysis

All data were statistically analysed 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 the same or Games-Howell test whenever variances were different. Significant differences were considered for $p<0.05$. When data did not follow a normal distribution, logarithmic or arcsin

transformation was carried out or non-parametric tests, such as Kruskal-Wallis, were used. The effects of the sources were evaluated by means of a two-way ANOVA, employing the different sources as fixed factors. Significant differences were considered when $p<0.05$.

4.4. RESULTS

4.4a. Growth and productive parameters

From 21 days of feeding until the end of the trial, body weight was significantly lowest in fish fed ZnAA/MnOx diet, followed by those fed ZnAA/MnAA diet (Table 4.3). Thus, supplementation with Zn chelated to amino acids reduced sea bream growth, whereas the inclusion of Zn as oxide (ZnOx/MnOx and ZnOx/MnAA) resulted in the highest growth at the end of the experiment (Table 4.3). Therefore, the two-way ANOVA showed a clear significant ($p<0.001$) effect of the Zn source on growth. On the contrary, Mn supplementation in both forms did not significantly affected growth ($p>0.05$) (Table 4.3).

Table 4.3 Body weight (g) of gilthead sea bream fed diets with different mineral sources for 0, 21, 42, 63 and 84 days

Days	C-	ZnOx/ MnOx	ZnAA/ MnAA	ZnAA/ MnOx	ZnOx/ MnAA	*p* value
0	8.2 ± 0.7	8.2 ± 0.8	8.2 ± 0.7	8.3 ± 0.7	8.2 ± 0.8	n.s.
21	13.3 ± 1.6^{b}	13.3 ± 1.6^{b}	13.1 ± 1.5^{ab}	13.0 ± 1.6^{a}	13.3 ± 1.6^{b}	0.000
42	20.8 ± 3.4^{ab}	21.1 ± 3.2^{ab}	20.7 ± 3.1^{ab}	20.6 ± 3.2^{a}	21.2 ± 3.3^{b}	0.002
63	31.9 ± 6.1^{ab}	32.2 ± 5.9^{ab}	31.4 ± 5.6^{ab}	31.1 ± 5.7^{a}	32.3 ± 6.1^{b}	0.005
84	44.2 ± 9.2^{a}	46.1 ± 8.8^{b}	45.0 ± 8.3^{ab}	44.2 ± 8.7^{a}	46.3 ± 9.3^{b}	0.000

Different letters in a row denote significant differences between groups fed different diets for a given feeding period (mean±SD, *n=3*). Two –way ANOVA revealed an effect ($p<0.001$); Zn source affected growth at 84 days. n.s.: non-significant.

FCR increased from the beginning of the trial to the end and ranged from 1.07-1.15 at day 21 up to 1.32-1.51 at day 84 (Table 4.4). Even though no statistical differences (p<0.05) were found between the different treatments at any given time, fish fed the diet with no Zn or Mn supplementation showed the highest FCR at the end of the trial (Table 4.4).

Table 4.4 FCR of gilthead sea bream fed diets with different mineral sources for 21, 42, 63 and 84 days

Days	C-	ZnOx/ MnOx	ZnAA/ MnAA	ZnAA/ MnOx	ZnOx/ MnAA	*p* value
21	1.09 ± 0.08	1.11 ± 0.07	1.15 ± 0.04	1.07 ± 0.03	1.13 ± 0.03	n.s.
42	1.27 ± 0.06	1.23 ± 0.03	1.26 ± 0.01	1.21 ± 0.10	1.27 ± 0.06	n.s.
63	1.21 ± 0.04	1.25 ± 0.08	1.28 ± 0.07	1.26 ± 0.09	1.28 ± 0.10	n.s.
84	1.51 ± 0.06	1.34 ± 0.04	1.37 ± 0.08	1.37 ± 0.24	1.32 ± 0.23	n.s.

4.4b. Biochemical analyses

No supplementation of Zn or Mn (diet C-) translated into a higher lipid deposition in whole body, whilst supplementation with Mn or Zn, particularly in diet ZnOx/MnOx, significantly (p<0.05) reduced lipid contents (Table 4.5). Besides, these fish fed ZnOx/MnOx showed a significantly (p<0.05) higher protein deposition (Table 4.5). Ash content was not significantly affected by the diets (Table 4.5). Two-way ANOVA showed a significant (p=0.004) interaction on the effect of Zn and Mn sources in lipid deposition; an effect of Zn sources (p=0.028) and an interaction between Zn and Mn sources (p=0.006) for proteins; and an effect of Mn sources (p=0.014) on ash (Table 4.5). Lipid peroxidation, as denoted by the results of TBARs content, was highest on fish fed diet ZnAA/MnOx, and lowest on those fed C- (Table 4.5).

Table 4.5 Final composition (% dw) and TBARs of whole fish of gilthead sea bream juveniles fed diets with different mineral sources for 84 days

	C-	ZnOx/ MnOx	ZnAA/ MnAA	ZnAA/ MnOx	ZnOx/ MnAA	*p* value
Lipid (% DW)	46.0±1.1c	37.7±4.3a	38.9±1.2ab	40.9±1.8b	41.6±2.8bc	0.001
Protein (%)	49.5±1.7ab	51.5±2.1b	47.6±1.3ab	47.4±4.4a	46.6±0.8a	0.014
Ash (%)	9.0±0.5	9.0±1.0	9.4±0.8	9.8±1.8	10.1±0.5	n.s.
Moisture (%)	68.2±0.8ab	68.8±0.8b	65.7±0.5a	66.7±1.2ab	66.7±0.5ab	0.019
TBARs (nmol/g dry weight)	118 ± 54a	137 ± 15ab	161 ± 50ab	228 ± 12b	189 ± 15ab	0.021

Different letters in a row denote significant differences between groups fed different diets for a given feeding period (mean±SD, $n=3$, $p<0.05$). Two –way ANOVA revealed an interaction (p=0.004); between Zn and Mn sources and lipids. There was an effect on proteins for the source of Zn (p=0.028) and an interaction between the sources of Zn and Mn (p=0.006) as observed by two-way ANOVA. Two-way ANOVA also showed an effect of the Mn sources on ash content (p= 0.014).

4.4c. Mineral analyses

Feeding diets ZnAA/MnAA, ZnAA/MnOx and ZnOx/MnAA significantly (P=0.00) increased Zn contents in whole body in comparison to C- (Table 4.6), whereas fish fed ZnAA/MnAA presented intermediate values. The two-way ANOVA analysis showed a stronger effect of Mn dietary form (P=0.028) on whole body Zn contents than the type of Zn (P=0.074). Thus, MnOx significantly reduced Zn contents in whole body, whereas MnAA did not reduced Zn deposition. Combined inclusion of ZnOx and MnAA led to the highest Zn body contents (P=0.031).

Mn whole body contents in fish fed the C- diet were not increased by the supplementation with neither MnOx or MnAA (Table 4.6), but there was a highly significant interaction between Zn type and Mn type (0.004), ZnOx and MnOx feeding leading to the lowest Mn

contents in whole body. Thus, when fish were fed MnOx, whole body contents of Mn were significantly (P=0.02) lower in fish fed ZnOx/MnOx than in those fed ZnAA/MnOx, namely ZnOx significantly reduced whole body Mn contents.

Mg, Ca and P whole body contents were significantly lowest in fish fed C- or ZnOx/MnOx (P=0.00) (Table 4.6). Feeding MnAA significantly (P<0.00) increased Mg, Ca and P contents in whole body, particularly when ZnOx was included in the diet (P=0.00) as denoted by the two-way ANOVA. Moreover, a significant correlation was found between the contents of Ca and P (R^2=0.99), Mg and Ca (R^2=0.97) and Mg and P (R^2=0.95) (P=0.000). However, there was no correlation between the Mn or Zn contents in diet or whole body and the body contents in Ca, P, or Mg (data not showed).

No significant differences in whole body mineral composition of K, Fe and Cu were observed among fish fed the different diets (Table 4.6).

As of the vertebrae mineral composition, there were no significant differences for any of the minerals analysed (Table 4.7).

Table 4.6 Whole body mineral composition of gilthead sea bream fed the experimental diets for 12 weeks (dry weight basis)

Whole body mineral composition		C-	ZnOx/MnOx	ZnAA/MnAA	ZnAA/MnOx	ZnOx/MnAA	One-way ANOVA (*p-value*)	Two-way ANOVA (*p-value*)		
								Zn	Mn	Zn x Mn source
Zn	(mg/kg)	12.10 ± 0.34a	17.07 ± 0.29ab	19.06 ± 0.60b	19.03 ± 1.06b	19.30 ± 0.07b	0.00	0.074	0.028	0.031
Mn	(mg/kg)	4.56 ± 0.26ab	4.32 ± 0.11a	4.48 ± 0.10ab	5.10 ± 0.37b	4.79 ± 0.08ab	0.02	0.117	0.625	0.004
Mg	(g/kg)	0.59 ± 0.01ab	0.58 ± 0.00a	0.61 ± 0.01bc	0.63 ± 0.01cd	0.66 ± 0.02d	0.00	0.706	0.002	0.000
Ca	(g/kg)	30.3 ± 0.3a	30.4 ± 0.4a	31.8 ± 0.2b	32.6 ± 0.3b	33.8 ± 0.1c	0.00	0.690	0.000	0.000
P	(g/kg)	15.7 ± 0.3a	15.9 ± 0.3a	16.5 ± 0.1b	17.0 ± 0.1b	17.6 ± 0.1c	0.00	0.836	0.001	0.000
K	(g/kg)	1.63 ± 0.2	1.69 ± 0.1	1.78 ± 0.2	1.92 ± 0.1	1.93 ± 0.1	0.14	0.689	0.578	0.065
Fe	(mg/kg)	7.99 ± 1.48	8.26 ± 0.88	8.77 ± 1.85	8.07 ± 1.82	7.90 ± 0.42	0.56	0.579	0.408	0.312
Cu	(mg/kg)	0.34 ± 0.03	0.35 ± 0.03	0.41 ± 0.01	0.36 ± 0.05	0.33 ± 0.00	0.08	0.053	0.383	0.102

Different letters in a row denote significant differences between groups fed different diets for a given feeding period (mean±SD, $n=3$, $p<0.05$).

Table 4.7 Vertebrae mineral composition of gilthead sea bream fed the experimental diets for 12 weeks (dry weight basis)

Vertebrae mineral composition		C-	ZnOx/MnOx	ZnAA/MnAA	ZnAA/MnOx	ZnOx/MnAA	One-way ANOVA (*p-value*)
Zn	(mg/kg)	360.7 ± 64.7	349.3 ± 71.0	378.0 ± 15.1	341.7 ± 52.4	377.3 ± 43.8	0.88
Mn	(mg/kg)	41.9 ± 1.5	41.7 ± 6.0	40.7 ± 1.6	44.9 ± 4.5	39.1 ± 2.3	0.45
Mg	(g/kg)	10 ± 0	10 ± 0	10 ± 0	10 ± 0	10 ± 0	0.11
Ca	(g/kg)	262 ± 0	262 ± 4	260 ± 1	261 ± 4	257 ± 8	0.67
P	(g/kg)	172 ± 2	170 ± 2	171 ± 1	169 ± 1	170 ± 2	0.46
K	(g/kg)	78 ± 1	77 ± 2	78 ± 1	75 ± 3	80 ± 4	0.26
Fe	(mg/kg)	212.7 ± 11.7	208.7 ± 8.0	218.0 ± 4.4	213.7 ± 23.4	215.3 ± 23.9	0.97
Cu	(mg/kg)	22.4 ± 5.8	18.0 ± 1.7	19.3 ± 3.6	18.4 ± 0.8	20.5 ± 2.7	0.62

Different letters in a row denote significant differences between groups fed different diets for a given feeding period (mean±SD, $n=3$, $p<0.05$).

4.4d. Gene expression

Expression of *cuznsod* presented no statistical differences between the treatments, however, fish fed ZnAA/MnOx presented a higher expression than fish fed the other diets (Table 4.8). *mnsod* expression was similar for all the treatments and non-significant (Table 4.8).

Table 4.8 Expression level of *cuznsod* and *mnsod* in liver of gilthead sea bream juveniles fed diets with different mineral sources for 12 weeks

Relative expression (2^-ΔΔCt)	C-	ZnOx/ MnOx	ZnAA/ MnAA	ZnAA/ MnOx	ZnOx/ MnAA	*p* value
cuznsod	1.00 ± 0.10	0.89 ± 0.08	1.09 ± 0.84	2.41 ± 0.71	1.63 ± 0.39	n.s.
mnsod	1.08 ± 0.49	1.21 ± 0.50	0.95 ± 0.37	1.23 ± 0.17	1.87 ± 1.12	n.s.

4.4e. Histology

Histological analyses of the livers evidenced there was no significant effect on liver steatosis by any of the dietary treatments. Overall, fish presented a mild steatosis with mean 1.7 ± 0.5.

4.5. DISCUSSION

Fish fed Zn oxide presented the highest growth, independently if they were fed Mn oxide or chelates, denoting the positive metabolic effect of Zn oxide on gilthead sea bream in the present study. These results are in agreement with the higher growth found previously in gilthead sea bream juveniles fed a mix of Zn oxide, Mn oxide and sodium Se in comparison to fish fed AA chelated forms of these minerals (Dominguez *et al.*, 2017). However, in other marine (European seabass, *Dicentrarchus labrax*, Fountoulaki *et al.*, 2010) or freshwater species (Nile tilapia, *Oreochromis niloticus*, do Carmo e Sá *et al.*, 2005), Zn oxide led to similar growth than organic forms. This lack of effect in the last two studies could be related to the high levels of Zn supplemented in both the European

seabass diets (over 250 mg Zn kg^{-1}) and the Nile Tilapia diets (150 mg Zn kg^{-1}) that would be sufficient to widely cover Zn requirements for growth in those species, and reduce the differences between both mineral sources. For instance, in Nile tilapia requirements have been described to be around 30-80 mg Zn kg^{-1} (Antony Jesu Prabhu *et al.*, 2016). In the present trial, Zn levels in the non-supplemented diet were much lower (49 mg Zn kg^{-1}), and insufficient to cover Zn requirements for gilthead sea bream juveniles (around 150 mg Zn kg^{-1} Dominguez et al, *submitted a*). In agreement, Zn whole body contents were lowest in fish fed the non-supplemented diet, whereas Zn supplementation in either the oxide or AA chelated form increased Zn whole body contents. Despite there were no significant differences in FCR between the treatments, at the end of the trial Zn and Mn supplementation tend to improve FCR, regardless of the source used, in agreement with the improved FCR found in several fish species when fed Mn or Zn (Antony Jesu Prabhu *et al.*, 2016).

The lowest lipid deposition and highest protein retention of fish fed both Zn and Mn oxides suggests the effectiveness of this source, since high lipid deposition and low protein may be a symptom of low Zn (Wu *et al.*, 2014) content or Mn deficiency (Chiba, 2004), and may be related to the negative effect of feeding diets with plant protein sources (Kaushik *et al.*, 2004). Lipid peroxidation is a result of oxidative damage, and can be measured by the measurement of MDA (Devasena *et al.*, 2001). TBARS have been extensively used as a method to evaluate the effects of compounds that can potentially affect lipid peroxidation such as copper, selenium, folic acid, vitamin E and DHA (Betancor *et al.*, 2013; Hoyle *et al.*, 2007; Izquierdo *et al.*, 2013; Lin and Shiau, 2009; Lin *et al.*, 2011 Shaw *et al.*, 2012). Interestingly, fish fed the diet with no Zn or Mn supplementation presented the lowest lipid peroxidation, while the rest of the diets presented intermediate values. Evidence of the effects of Zn supplementation in reducing

lipid peroxidation can be found in Jian carp, where increasing Zn supplementation reduced the levels of MDA in several tissues including intestine, hepatosomatic tissue and muscle (Feng *et al.*, 2011). Experiments conducted in rats exposed to chlorpyrifos, an insecticide which in high doses can produce increased oxidative stress and enhanced TBARS levels, had a reduced hepatotoxic effect when supplemented with Zn, and in consequence a reduced lipid peroxidation (Goel *et al.*, 2005), even reducing TBARS to levels similar to control doses (Mansour and Mossa, 2009). In the present study, lipid peroxidation, as denoted by the TBARS analysis, was increased on fish fed diet ZnAA/MnOx, a result that matches with the lowest growth in fish fed this diet. Moreover, this fish also showed the highest content in whole body Mn. In fact, Mn contents in the non-supplemented diet (30 mg Mn kg^{-1}) matched the optimum dietary Mn levels described in other trials (19 mg Mn kg^{-1}, Dominguez *et al.*, *submitted a*).

Interestingly, when Mn was supplemented as Mn oxide, it inhibited deposition of Zn from Zn oxide in whole body, denoting the competition of the inorganic forms of both minerals. On the contrary, supplementation of Mn in the AA chelated form did not negatively affect Zn incorporation, suggesting that the inhibition of Zn incorporation by Mn oxide could be related to the competition during absorption. Likewise, in fish fed Mn oxide, supplementation with Zn oxide reduced whole body Mn contents in comparison to fish fed ZnAA, suggesting again the competition between inorganic forms of both minerals during absorption. The degree of absorption of both elements may depend on its chemical form, interactions with different elements, developmental stage, etc. They may cross the cell membrane in a passive form according to the concentration gradient or in an active one against the concentration gradient. Competition between Mn and Zn could be also find at other physiological levels. Regarding transport, both Mn and Zn may be transported by α2-macroglobulin. Also, metallomethioneins, responsible of maintaining

Zn and Cu homeostasis, have a high affinity for Mn. Besides, in complex compounds containing these minerals, covalent bonds are more stable with Mn^{2+} than with Zn^{2+} according to Irving -William series (Długaszek, 2019), that could lead to a displacement of Zn by Mn in those compounds. However, this was not the case in the present study, since when fish were fed both minerals in the AA chelated form, Mn did not reduce Zn whole body contents, further supporting the hypothesis of a competition at the absorption level. Mn is absorbed through the gut by passive diffusion or active transport, mainly by the divalent metal transporter 1 (DMT1), however its transporters are not Mn-specific, but also regulate Fe, Cu, Zn and other metals uptake. Therefore, Mn may compete with Zn for these transporters (Chen *et al.*, 2018). Reduction of Zn whole body contents by Mn oxide, agrees well with the results found in other species (Liu *et al.*, 2017; Nie *et al.*, 2016), while in other ones there was no effect (Tan *et al.*, 2012; Zhang *et al.*, 2016), or even tended to increase the content in a precise level of supplementation (Ye *et al.*, 2009). These differences could be not only species specific but also related to the levels and molecular form to supplement these minerals.

Feeding MnAA, particularly in combination to ZnOx, increased Mg, Ca and P contents in whole body, suggesting that MnOx competes with Ca, Mg or P, whereas MnAA does not. Mn and Zn may interact in a synergistic or antagonistic manner with Mg and Ca, at different physiological levels including absorption, transmembrane transport, biochemical processes, accumulation in tissues, or excretion (Długaszek, 2019). Since, Ca, Mg or P whole body contents were not related to the whole body contents of Mn or Zn, such competition between Mn fed as MnOx and Mg, Ca or P may occur at an absorption level. This would be in agreement with the reduction in Ca absorption observed in other fish species (Liang *et al.*, 2015). This would be further supported by the fact that when ZnOx, which also competes with MnOx at an absorption level, was

supplemented in the diet, the levels of Ca, Mg and P were increased. Mn^{2+} and Mg^{2+} bond with similar ligands, but Mn has a higher affinity for N, which is the atom donor in ligands, than Mg (Długaszek, 2019). If Mn oxide would be responsible for a competition with either Mg or Ca, this may affect both elements, as well as P, since their concentrations in body fluids and tissues correlate positively in vertebrates (Długaszek, 2019). In one hand, Ca and Mg are s-block elements with similar chemical properties that are mainly stored in bone, scales and teeth. Mg and Ca have a clear synergistic relationship at an intestinal absorption level. For instance, Mg facilitates Ca absorption by activating vitamin D3 (Długaszek, 2019). Besides, their proportions are regulated by homeostatic processes. In the other hand, since fish must retain a specific Ca to P ratio, alterations in Ca content may also regulate the P content (Lall, 2002).

As for vertebrae mineral composition, there was no significant difference between treatments. Whole body and vertebrae Zn and Mn content has been used as an indicator of mineral retention in several species including Atlantic salmon (*Salmo salar*), carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), channel catfish (*Ictalurus punctatus*), cobia (*Rachycentron canadum*), tilapia (*Oreochromis niloticus*), grouper (*Epinephelus coloides*), rainbow trout (*Oncorhynchus mykiss*) (Antony Jesu Prabhu *et al.*, 2016). On the other hand, from our previous experience, vertebral Mn remains unaltered independent of the different sources, while vertebral Zn is reduced when fish are not supplemented with this mineral (Dominguez *et al.*, 2017). In the present trial, there was no effect on vertebrae mineral composition, but a lower Zn content was found in whole body of fish fed the control diet without Zn supplementation, suggesting Zn supplementation is necessary to increase Zn levels.

Despite no significant differences between treatments, results from liver *cuznsod* gene expression analyses showed a similar trend as to those obtained in TBARS quantification,

where diet ZnAA/MnOx presented the highest expression, followed by diet ZnOx/MnAA, while diets C-, ZnOx/MnOx and ZnAA/MnAA presented the lowest expression. *cuznsod* intervenes in reducing the oxidative damage produced by free radicals, compounds that are naturally formed during lipid peroxidation (Halliwell and Gutteridge, 2000). High lipid peroxidation is paired to reduced *cuznsod* activity (Feng *et al.*, 2011; Wu *et al.*, 2014). The higher TBAR levels observed on fish fed diet ZnAA/MnOx was paired to a higher *cuznsod* expression as an attempt to reduce oxidative damage produced as a result of lipid peroxidation. Results for liver *mnsod* expression showed no differences between the dietary treatments. In rainbow trout, *mnsod* activity was increased in an attempt to compensate for *cuznsod* decreased activity (Hidalgo and Exposito, 2002), however this was not the case in the present study.

Results from histological analyses of the liver revealed a mild steatosis throughout every treatment, but no differences were found between them. Steatosis can appear when hepatocytes are unable to oxidize fatty acids from dietary lipids, or in the case of inadequate protein synthesis, thus leading to an increase in the deposition of triglycerides as hepatic vacuoles (Tacon, 1996). In gilthead sea bream, this condition is also related to the type of oil used in the feed formulation, since the oxidation rates vary between the oil sources (Caballero *et al.*, 2004), nevertheless, in the present trial, the main lipid source was the same in all the treatments.

In summary, Zn supplementation in the form of Zn oxide, improved growth, body composition and mineral retention of gilthead sea bream fed diets containing 15% FM and vegetable meals. On the contrary supplementation with high levels of Mn oxide, together with AA chelated Zn, lead to high whole body contents of Mn, did not improve growth and increased oxidative risk.

CHAPTER 5: DIETARY MANGANESE LEVELS FOR GILTHEAD SEA BREAM (*Sparus aurata*) FINGERLINGS FED DIETS HIGH IN VEGETABLE INGREDIENTS

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Keywords

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

5.1. ABSTRACT

Manganese (Mn) is a cofactor for essential metalloenzymes involved in the development of bone and preventing oxidative damage that tends to be in higher concentrations in ingredients of terrestrial origin. Mn requirements have been established for several finfish but not for 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 (FM: 10%, FO: 6%). The diets were supplemented with 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. At the end of the trial, samples were taken for biochemical, mineral, histological, gene expression and X-ray analyses. Performance parameters including feed intake, thermal growth coefficient and feed conversion ratio were calculated.

After the feeding trial, fish almost tripled their weight, but dietary Mn levels did not affect growth, productive parameters or survival. The high FM 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. However, increase of Mn contents beyond 30 mg Mn kg^{-1} down-regulated *mnsod* expression. Expression of *cat* gene was not affected. Whole body, liver and vertebrae mineral contents were not affected by Mn supplementation. Similarly body lipid composition, protein and ash were neither affected by the dietary Mn. Morphological characteristics of liver also remained unaltered with the different dietary Mn levels, as so occurred with the occurrence of skeletal anomalies.

Overall, these results suggest that the Mn 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.

5.2. INTRODUCTION

Substitution of FM and FO with 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 (ARRAINA, 2015). 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 including the MnSOD, which intervenes preventing the initiation of the radical chain reaction (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*., 2014). 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 stablishes 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 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 (Alm-Eldeen *et al*., 2018; Kaur *et al*., 2018; Krishnani *et al*., 2003).

Manganese requirements have been described in several species including yellow catfish (5.5-6.4 mg Mn kg^{-1}, Tan *et al*., 2012), cobia (21.72 mg Mn kg^{-1}, Liu *et al*., 2013), hybrid grouper (*Epinephelus lanceolatus* × *E. fuscoguttatus*, 12.70 mg Mn kg^{-1}, Liu *et al*., 2017), rainbow trout (19 mg Mn kg^{-1}, Satoh *et al*., 1991) and common carp (13-15 mg Mn kg^{-1}, Satoh *et al*., 1992). However, little is known about Mn nutrition in gilthead sea bream juveniles (Domínguez *et al*., 2017). Only few studies have addressed the effect of dietary Mn on gilthead sea bream growth performance. 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 (Domínguez *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 Zn or Se contents as it was later demonstrated (Domínguez *et al*., *submitted a, b*). 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 (Domínguez *et al*., *submitted c*). However, in comparison to amino acid chelated Mn, Mn oxide up-

regulated the expression of superoxide dismutase gene (*mnsod*) (Domínguez *et al*., *submitted,* c). 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 (Domínguez *et al*., *submitted,* a). Since in all these studies Mn was supplemented together with other minerals, the results were not conclusive. 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.

5.3. 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.

5.3a. 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 5.1). 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 5.1 Ingredients and analysed Mn content of the experimental diets supplemented with increasing levels of Mn and fed to gilthead sea bream juveniles for 42 days

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
Analysed Mn (mg kg^{-1})	19.00	27.00	30.00	41.00	66.00

*Micronutrient premix: methionine (10.6 g/kg), lysine (28.5 g/kg), phosphate (0.67%), vitamin premix (0.18%) and mineral premix excluding Mn (0.11%), 122 mg Fe kg^{-1}, 6.6 mg Cu kg^{-1}, 128 mg Zn kg^{-1} and 0.9 mg Se kg^{-1}.

5.3b. Fish and experimental conditions

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 ± 0.4ºC, 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 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 SGR, TGC and 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 (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.

5.3c. Gene expression

i. 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.

ii. 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.

iii. Quantitative PCR

The nucleotide sequences of primers used in this study are reported in Table 5.2. 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 $2^{-\Delta\Delta Ct}$ method was applied to analyse the relative changes in gene expression.

Table 5.2 Sequences of primers used for gene expression analysis

Full gene name	Gene	Nucleotide sequence (5'-3')
Beta actin	*bact*	FW: TCTGTCTGGATCGGAGGCTC
		RV: AAGCATTTGCGGTGGACG
Ribosomal protein 27a	*rpl27*	FW: ACAACTCACTGCCCCACCAT
		RV: CTTGCCTTTGCCCAGAACTT
Mn superoxide dismutase	*mnsod*	FW: AGTGCCTCCTGATATTTCTCCTCTG
		RV: CCTGACCTGACCTACGACTATGG
Catalase	*cat*	FW: ATGGTGTGGGACTTCTGGAG
		RW: AGTGGAACTTGCAGTAGAAAC

5.3d. Biochemical 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, Lees & Stanley (1957) and ash by combustion in a muffle furnace at 600ºC 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.

5.3e. 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 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 analysed by pair evaluators in a 0-3 scale, where 0 was absence of observation and 3 presence in most of the liver.

5.3f. Skeletal anomalies

X-Ray analyses were conducted using a 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).

5.3g. Statistical analyses

All data were statistically analysed 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 $p<0.05$.

5.4. RESULTS

5.4a. Growth and productive parameters

Fish accepted well the feed from the beginning of the trial and there were no effects of the dietary Mn on survival rate which was very high (99.3±1.4%). Dietary Mn did not had a significant effect on any of the productive parameters including weight, weight gain, SGR, FCR or FE (Table 5.3).

Table 5.3 Growth performance and feed utilization in gilthead sea bream fed increasing contents of Mn for 42 days

Dietary Mn (mg kg^{-1})	Mn19	Mn27	Mn30	Mn41	Mn66
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.

5.4b. Gene expression

Increase in dietary Mn levels over 30 mg Mn kg^{-1} down-regulated *mnsod* expression that was significantly lowest in fish fed 66 Mn kg^{-1} (Table 5.4). 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 doubled that of fish fed any of the other diets (Table 5.4).

Table 5.4 Hepatic gene expression analyses of gilthead sea bream fed increasing levels of dietary Mn for 42 days

Dietary Mn (mg kg^{-1})	Mn19	Mn27	Mn30	Mn41	Mn66
mnsod	1.02 ± 0.26^{b}	0.84 ± 0.02^{ab}	1.00 ± 0.10^{b}	0.59 ± 0.07^{ab}	0.41 ± 0.14^{a}
cat	1.08 ± 0.47	0.55 ± 0.02	0.35 ± 0.01	0.43 ± 0.06	0.40 ± 0.14

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

5.4c. Biochemical analyses

Mineral analyses of whole fish, liver and vertebrae showed no significant effects of dietary Mn on the mineral concentrations on the different tissues (Table 5.5). However, vertebral Mn content followed a linear regression in relation to dietary Mn ($Y=23.996+0.060X$, $p=0.01$, $R^2=0.66$).

Table 5.5 Whole body and liver Mn content of gilthead sea bream fed increasing dietary contents of Mn for 42 days

Dietary Mn (mg kg^{-1})	Mineral	Mn19	Mn27	Mn30	Mn41	Mn66
	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
Whole fish	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
	Mn	25.0±1.7	26.3±1.5	25.0±0.0	26.3±1.2	28.0±1.0
	Cu	0.72±0.11	0.66±0.03	0.63±0.00	0.69±0.09	0.68±0.02
Vertebrae	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.

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 5.6).

Table 5.6 Biochemical composition (% fresh weight) in whole body of gilthead sea bream fed increasing contents of Mn for 42 days

Dietary Mn (mg kg^{-1})	Mn19	Mn27	Mn30	Mn41	Mn66
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

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

5.4d. Histology

Histological analyses of hepatic tissue of gilthead sea bream 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 5.7).

Table 5.7 Hepatic histological analyses of gilthead sea bream fed increasing levels of dietary Mn for 42 days

Dietary Mn (mg kg^{-1})	Mn19	Mn27	Mn30	Mn41	Mn66
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

5.4e. Skeletal anomalies

At the end of the feeding trial the prevalence of skeletal anomalies observed in gilthead sea bream juveniles was not significantly affected by dietary Mn levels (Table 5.8).

Table 5.8 Prevalence of skeletal anomalies (%) in sea bream fed increasing levels of dietary Mn

Dietary Mn (mg kg^{-1})	Mn19	Mn27	Mn30	Mn41	Mn66
Total anomalies	64±10	64±6	54±6	54±15	59±21
Pre-haemal lordosis	47±13	41±13	45±2	34±13	42±25
Pre-haemal vertebral fusion	6±0	9±4	0±0	0±0	0±0
Pre-haemal vertebral anomaly	10±5	11±2	0±0	0±0	18±0
Haemal lordosis	6±0	12±11	6±0	5±1	12±1
Haemal partial vertebral fusion	6±0	0±0	0±0	5±0	0±0
Caudal anomalous vertebrae	0±0	0±0	9±4	8±4	12±0
Anomalous maxillary and/or pre-maxillary	14±7	15±9	6±0	12±10	26±8

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

5.5. DISCUSSION

As an essential mineral, Mn function in the body has been amply described in several fish species (Halver and Hardy, 2002; Watanabe *et al*., 1997; NRC, 2011; Antony Jesu Prabhu *et al*., 2016), however the requirements for gilthead sea bream juveniles have not yet been addressed. Due to its essentiality, dietary Mn levels may exert an important effect on growth and feed utilization in several fish species including yellow catfish (5.5-6.4 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 (19 mg Mn kg^{-1}, Satoh *et al*., 1991) and common carp (13-15 mg Mn kg^{-1}, Satoh *et al*., 1992). Results from the present trial

showed that increase in dietary Mn 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} is sufficient to cover Mn 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) and 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 Mn. Even when semi-purified diets were used (Satoh *et al*., 2001), the main ingredient was FM, which has lower Mn 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}) (ARRAINA, 2015). The results indicate that in novel diets with high FM replacement by terrestrial plant ingredients basal levels of Mn may be enough to cover gilthead sea bream requirements for growth.

Since Mn is an essential metal that forms part of *mnsod*, intervenes in preventing the initiation of the 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 Mn requirements in hybrid tilapia (Lin *et al*., 2008a) and yellow catfish (Tan *et al*., 2012), where fish fed basal purified or semi-purified diets without Mn supplementation (2.89 and 3.1 mg Mn kg^{-1} respectively) presented lower hepatic MnSOD activity than fish fed diets supplemented with Mn. In the present trial *mnsod* expression decreased with increasing dietary Mn supplementation over 30 mg Mn kg^{-1} following a broken line model that suggested an optimum level of 44.1 mg Mn kg^{-1}. 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 this fish.

Tissue contents in Mn for vertebrae and whole body have been described as biomarkers to assess Mn requirements in 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), orange-spotted grouper (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*., 2008a) and rainbow trout (19 mg Mn kg^{-1}, Satoh *et al*., 1991). Results from the present trial did not show a significant difference in mineral content in whole fish or liver of sea bream, with respect to the concentrations of the minerals studied, which included Mn, Cu, Fe, Zn and Se. Despite being considered as the defining criterion for Mn, the content in vertebrae for this mineral did not show significant differences between fish fed dietary Mn. However there was a positive correlation between dietary Mn levels and its content in vertebrae, defined by a linear regression ($Y=23.996+0.060X$, $p=0.01$, $R^2=0.66$). This suggests, as in other studies, that Mn deposition in vertebrae is highly responsive to dietary Mn (Antony Jesu Prabhu *et al*., 2016).

Interactions between Mn and other minerals have been observed in several species including cobia (Nie *et al*., 2016), orange-spotted grouper (Ye *et al*., 2009), hybrid grouper (Liu *et al*., 2017), large yellow croaker (Zhang *et al*., 2016), where increasing the dietary content of Mn tended to alter the mineral composition of whole body, liver and vertebrae for Cu, Fe and Zn. Cu levels appeared to increase with Mn supplementation in hybrid grouper (Liu *et al*., 2017) and cobia (Nie *et al*., 2016), while remained unaffected in a similar way as the present trial in yellow catfish (Tan *et al*., 2012), orange-spotted grouper (Ye *et al*., 2009) and large yellow croaker (Zhang *et al*., 2016). Some authors

consider these effects to be due to variations in intestinal Mn absorption or dietary sources of Mn (Liu *et al*., 2017).

Fe concentration was reduced in whole body with increasing levels of dietary Mn in orange-spotted grouper (Ye *et al*., 2009), yellow catfish (Tan *et al*., 2012) and hybrid grouper (Liu *et al*., 2017). In liver of cobia, the levels of Fe increased with increasing dietary Mn (Nie *et al*., 2016), while hybrid grouper Fe hepatic levels tended to increase and then decrease with increasing levels of Mn supplementation (Liu *et al*., 2017). Vertebral Fe proved to be the least responsive of the tissues, since only hybrid grouper and cobia were affected, and opposite trends were observed in hybrid grouper (Fe levels tended to reduced and then increase with dietary Mn, Liu *et al*., 2017) and cobia (Fe levels increased and then decreased with Mn supplementation, Nie *et al*., 2016). Fe and Mn interactions in these studies may be due to competition for the transporters that both minerals share, which include ferroportin and DMT1 (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 Fe supplementation in the present trial compared to the other studies, which in turn resulted in a lower Fe tissue content, thus reducing the competition for the common transporters.

In some studies, Zn was reduced with increasing dietary Mn (Liu *et al*., 2017; Nie *et al*., 2016), while in other ones there was no effect (Tan *et al*., 2012; Zhang *et al*., 2016), or even tended to increase the content in a precise level of supplementations (Ye *et al*., 2009). The authors argued that the effects of Zn on vertebrae mineralization were either due to its properties as a divalent cation acting on nucleation and mineral accumulation, or as a cofactor of enzymes (Liu *et al*., 2017).

Another possible explanation to the different interactions between Mn and Cu, Fe and Zn in the species described and gilthead sea bream may be the use higher levels of these

minerals employed in trials for other fish species when compared to the levels in the present trial (6.6 mg Cu kg^{-1}; 122 mg Fe kg^{-1}; 128 mg Zn kg^{-1}). Whatever the case may be, the mineral contents in gilthead sea bream tissues were not affected by Mn supplementation.

Dietary Mn did not had a significant effect on whole body lipid, protein or ash content. Cobia fed purified diets and increasing levels of Mn 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 Mn (3.1-19.5 mg Mn kg^{-1}), where whole body lipid tended to decrease with increasing levels of Mn (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 Mn resulted in an increased weight, thus altering the protein and lipid content of the whole body of these species.

Dietary Mn did not alter hepatic morphology of gilthead sea bream juvenile. Fish submitted to large amounts of water-borne Mn and other heavy metals presented a series of hepatic alterations in morphology. Those apparently more specific to Mn toxicity included pycnotic degeneration of hepatocytes in barramundi (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 Mn intoxications,

while no hepatic alterations are mentioned in trials conducted on fish with dietary Mn nor in the present trial.

Manganese deficiencies may course with skeletal anomalies such as dwarfism (Satoh *et al*., 1983, 1987; Yamamoto *et al*., 1983; Lall *et al*., 2002). Common carp presented these symptoms when fed diets with Mn supplementation below 6 µg/g Mn, and the authors concluded that addition of 10 µg/g Mn was sufficient to reduce this prevalence to 0% (Satoh *et al*., 1983, 1987). Red sea bream larvae fed artemia enriched with Mn (12-42.8 µg/g Mn) presented a lower prevalence of skeletal anomalies than those fed a control artemia-based diet without Mn (7.81 µg/g Mn), evidencing the need of Mn supplementation in larval culture of this species (Nguyen *et al*., 2008). Rainbow trout fed diets without Mn supplementation (3.8-4.4 µg/g Mn) presented dwarfism, while addition of Mn (22.5-24.5 µg/g Mn) reduced the prevalence to 0% (Yamamoto *et al*., 1983). On the other hand rainbow trout fed a semi-purified diet containing 5.1-23.9 µg/g Mn did not present dwarfism (Satoh *et al*., 1991), suggesting the requirement of Mn for this species to reduce the prevalence of dwarfism must be in a range between those described by Yamamoto *et al*. (1983) and Satoh *et al*. (1991), namely 4.5-5.1 µg/g Mn. Thus, the absence of dwarfism or an effect of dietary Mn levels on skeletal anomalies may be due to the fact that the levels presented in the present trial (above 19 mg Mn kg^{-1}) are well above those described as required for common carp (10 µg/g Mn), red sea bream larvae (12-42.8 µg/g Mn) and rainbow trout (4.5-5.1 µg/g Mn).

In summary, the presence of Mn in higher concentrations in plant ingredients than animal sources suggests that practical diets based on plant ingredients may contain sufficient Mn to cover the requirements for gilthead sea bream fingerlings. In the present study, markers for growth, feed utilization, tissue mineral content, whole body chemical composition, hepatic morphology or prevalence of skeletal anomalies were not affected by Mn

supplementation, thus suggesting that the Mn 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}).

CHAPTER 6: OPTIMUM SELENIUM LEVELS IN DIETS HIGH IN PLANT BASED FEEDSTUFFS FOR GILTHEAD SEA BREAM (*Sparus aurata*) FINGERLINGS

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6.1. ABSTRACT

Substitution of fish meal (FM) and fish oil (FO) ingredients by plant protein and oil sources can modify selenium (Se) levels in feeds. Se plays an important role in the anti-oxidative defense by forming part of selenoproteins. Despite being a species of major interest for Mediterranean aquaculture, the Se requirements of gilthead sea bream (Sea bream) are not accurately determined, particularly when fed low FM-FO diets. Therefore, this study was conducted to define Se supplementation levels in low FM-FO practical diets for sea bream fingerlings.

A plant-based diet (FM 10% and FO 6%) containing 0.45 mg Se kg^{-1} 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^{-1} diet, supplied as sodium selenite. Fingerlings of sea bream, weighing 12.6 ± 1.4 g were distributed into 15 tanks in triplicate groups per diet and fed until apparent satiation three times daily for 42 days. Se supplementation up to 1.00 mg Se kg^{-1} significantly improved the growth of juvenile sea bream, whereas further increase up to 1.7 mg Se kg^{-1} diet reduced growth, in terms of final standard length and body weight. Body composition analyses revealed an increased lipid deposition in whole body of fish fed 0.86 mg Se kg^{-1}, while protein and ash were not significantly affected by dietary Se. Supplementation up to 0.86 mg Se kg^{-1} proportionally down-regulated of glutathione peroxidase gene (*gpx-1a*) expression, while further elevation of Se up-regulated it. Both glucocorticoid receptor gene (*gr*) and catalase (*cat*) genes were significantly up-regulated in fish fed 1.70 mg Se kg-1, suggesting an increased oxidative stress caused by excessive dietary Se levels.

In conclusion, the results of this study suggest that the optimum dietary levels of sodium selenite in diets with 10% FM with basal levels of 0.45 mg Se kg^{-1} are around 0.94 mg Se kg^{-1} to promote growth of gilthead sea bream juveniles. On the contrary, dietary levels of

1.7 mg Se kg^{-1} were found to be excessive and caused growth reduction, increased catalase expression and hydraulic degeneration in the liver.

6.2. 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 *et al.*, 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 decades. 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 *et al.*, 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 sulfur 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 *et al.*, 2017). Se toxicity curses with reduced growth and feed efficiency, increased oxidative stress

mortality, increased skeletal anomalies, edema, 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 hematocrit (Bell *et al.*, 1987; Berntssen *et al.*, 2018; Betancor *et al.*, 2012; Choi *et al.*, 2015; Gatlin, 1984; Lin and Shiau 2005; Pacitti *et al.*, 2015; Saleh *et al.*, 2014; Schultz and Hermanutz, 1990; Tashjian *et al.*, 2006; Watanabe *et al.*, 1997; Zee *et al.*, 2016b).

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 $kg^{-1,}$ respectively (Le and Fotedar, 2014a,b; Lin, 2014; Liu *et al.*, 2010; Mansour *et al.*, 2017). Other species do not require Se supplementation, as cutthroat trout (*Oncorhynchus clarki bouvieri*) (Hardy *et al.*, 2010), but most species typically require between 0.12-1.85 mg Se kg^{-1}. Catfishes belong to those species with the lowest requirements (*Clarias gariepinus* and *Ictalurus punctatus*; Abdel-Tawwab *et al.*, 2007; Wang and Lovell, 1997), while carps (*Carassius auratus gibelio*, *Ctenopharyngodon idellus* and *Cyrpinus carpio*; Ashouri *et al.*, 2015; Liu *et al.*, 2018; Zhu *et al.* 2016), hybrid striped bass (*Morone chrysops* × *M. saxatilis*; Jaramillo *et al.*, 2009) and largemouth bass (*Micropterus salmoide*; Zhu *et al.*, 2012) have the highest ones. As for sparids, requirements for red sea bream (*Pagrus major*), a species close to gilthead sea bream, are 1.34 mg Se kg^{-1} (Dawood *et al.*, 2019), whereas optimum Se dietary levels for gilthead sea bream are not accurately determined yet.

Se 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 *et al.*, 2016, Khan *et al.*, 2017).

Se forms part of selenoproteins, being the teleost fishes the organisms with the highest number of them, up to thirty-eight in zebrafish (*Danio rerio*) (Mariotti *et al.*, 2012). The glutathione peroxidase (GPx) is amongst 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 and Meyer, 2008; Holley *et al.*, 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 system 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 and 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 *et al.*, 2011; Mansour *et al.*, 2017; Misra *et al.*, 2012; Penglase *et al.*, 2014). Oxidative stress may also alter transcription factors, such as glucocorticoid receptors (*gr*) (Di Giulio & Meyer 2008, Esposito *et al.*, 1998, Olsvik *et al.*, 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 *et al.*, 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.

6.3. 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.

6.3a. Diets

In previous studies, gilthead sea bream fed graded levels of Se and other micronutrients showed best Se retention and enhanced anti-oxidant defenses when fish was fed at 0.77 mg Se kg^{-1} (Dominguez *et al.*, *submitted a*), 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 (10%) and FO (6%). Five different experimental diets were then produced by supplementing sodium selenite (Na_2SeO_3), to contain 0.45, 0.68, 0.86, 1.0 and 1.7 mg Se kg^{-1} diet (Table 6.1). 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 6.1 Ingredient composition and analysed Se contents of the experimental diets supplemented with increasing levels of sodium selenite

Ingredient (%)	Se0.45	Se0.68	Se0.86	Se1.00	Se1.70
Linseed oil	0.82	0.82	0.82	0.82	0.82
Wheat	11.69	11.68	11.68	11.68	11.66
Corn gluten	15.00	15.00	15.00	15.00	15.00
Wheat gluten	21.66	21.66	21.66	21.66	21.67
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
Analysed Selenium (mg kg^{-1})	0.45	0.68	0.86	1.00	1.70

*Micronutrient premix includes: methionine (10.6 g/kg), lysine (28.5 g/kg), phosphate (0.67%), vitamin premix (0.18%) and mineral premix excluding Se (0.11%), 122 mg Fe kg^{-1}, 6.4 mg Cu kg^{-1}, 126 mg Zn kg^{-1} and 32 mg Mn kg^{-1}.

6.3b. Fish and experimental conditions

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 ± 0.4ºC, 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 hours fasting. During samplings, fish were caught and introduced into an anesthetic tank containing clove oil (Guinama S.L.U., Valencia, Spain) 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 -80ºC until the analysis was conducted. Twenty fish per tank were sampled for radiographic assessment.

6.3c. Biochemical analyses

Biochemical analysis of diets and whole fish was conducted by following standard procedures (AOAC, 2000). Crude lipid was extracted according to Folch *et al.* (1957) and ash was determined 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 calculated after drying the sample in an oven at 105 ºC until reaching constant weight. The evaluation of the mineral content was conducted by means of an inductively coupled plasma mass spectrometry (iCAPQ ICP-MS).

6.3d. Gene expression

i. 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.

ii. 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 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.

iii. Quantitative PCR

The nucleotide sequences of primers used in this study are reported in Table 6.2. 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. 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, 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 $2^{-\Delta\Delta Ct}$ method was applied to analyse the relative changes in gene expression.

Table 6.2 Sequences of primers used for gene expression analysis

Full gene name	Gene	Nucleotide sequence (5'-3')
Beta actin	*bact*	FW: TCTGTCTGGATCGGAGGCTC
		RV: AAGCATTTGCGGTGGACG
Ribosomal protein 27a	*rpl27*	FW: ACAACTCACTGCCCCACCAT
		RV: CTTGCCTTTGCCCAGAACTT
Glutathione peroxidase	*gpx1a*	FW: GCTTTGAGCCAAAGATCCAG
		RV: CTGACGGGACTCCAAATGAT
Catalase	*cat*	FW: ATGGTGTGGGACTTCTGGAG
		RW: AGTGGAACTTGCAGTAGAAAC
Glucocorticoid receptor	*gr*	FW: GGGCTGGATGGAAGAACGACA
		RW: ACACCGAAAGCACTGAGGAGG

6.3e. 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, 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 3 µm 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 steatosis, including lipid accumulation, broken cell margin or nuclei displacement to cell periphery, as well as sinusoid dilatation. This 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, hydropic degeneration were observed in some fish and its presence was used to determine the percentage of fish with this incidence in each tank.

6.3f. Statistical analysis

All data were statistically analysed 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 the same or Games-Howell test whenever variances were different. Significant differences were considered for $p < 0.05$. When data did not follow a normal distribution, logarithmic or arcsin transformation was carried out or non-parametric tests, such as Kruskal-Wallis, were used. Two different regression models (broken-line and quadratic regressions analysis) (Robbins *et al*., 1979; Lin *et al*., 2010) were applied to understand the effect of dietary Se on different parameters and estimate gilthead sea bream requirements for dietary Se.

6.4. RESULTS

6.4a. 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 6.3). At the end of the trial, elevation of the dietary

Se levels up to 1.0 mg Se kg^{-1} significantly improved standard length, whereas further increase significantly reduced this growth parameter. Equally, body weight was significantly increased by elevation of dietary Se levels up to 1.0 mg Se kg^{-1}, whereas further increase reduced body weight (p=0.021) (Table 6.3). Similar tendencies were found for other growth parameters studied (SGR, weight gain or TGC), despite no significant differences were detected (Table 6.3). Feed utilization was not significantly affected by dietary Se levels and showed an average value of 0.88±0.02 for all fish groups.

Table 6.3 Productive parameters data of gilthead sea bream fed increasing dietary Se levels for 42 days

Analysed dietary Se (mg kg^{-1})	Days	0.45	0.68	0.86	1.00	1.70
Survival rate (%)	42	98±4	97±3	100±0	100 ±0	98±2
Standard length (cm)	42	11.3±0.5a	11.5±0.5ab	11.6±0.5ab	11.7±0.4b	11.3±0.6a
Body Weight (g)	18	21.2 ± 2.9a	21.8 ± 2.8a	21.9 ± 2.8ab	22.5 ± 2.9b	21.8 ± 2.8a
	42	31.6±4.7a	32.8±4.3a	33.4±4.4ab	35.7±4.0b	31.9±4.8a
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
Survival rate (%)	42	98±4	97±3	100±0	100 ±0	98±2
Standard length (cm)	42	11.3±0.5a	11.5±0.5ab	11.6±0.5ab	11.7±0.4b	11.3±0.6a

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

6.4b. Biochemical analyses

At the end of the trial, increase in dietary Se up to 0.86 mg Se kg^{-1} significantly increased whole body lipids whereas further Se elevation reduced this biochemical parameter (Table 6.4). Moisture content significantly increased in fish fed the highest dietary Se levels (1.7 mg Se kg^{-1}), particularly in relation to those fed 1.0 mg Se kg^{-1} (Table 6.4).

Dietary Se supplementation did not significantly affected whole body protein and ash content (Table 6.4).

Table 6.4 Biochemical composition (% fresh weight) in whole body of gilthead sea bream fed increasing contents of selenium for 42 days

Analysed dietary Se (mg kg^{-1})	0.45	0.68	0.86	1.00	1.70
Lipids	11.9±0.9a	12.3±0.7a	14.0±0.7b	11.8±0.9a	12.6±0.9ab
Protein	17.1±0.6	17.2±1.1	16.9±0.7	17.4±0.8	16.7±0.6
Ash	3.5±0.3	3.2±0.6	3.3±1.0	3.6±2.1	4.0±1.0
Moisture	66.3±0.45b	66.5±0.7b	66.4±0.6b	65.9±1.1a	67.4±0.4c

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

Se content in whole fish increased not significantly with increasing Se supplementation for whole body that reached a plateau at 1.0 mg Se kg^{-1} (Table 6.5). 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^{-1} and the lowest in those fed 0.45 mg Se kg^{-1} (Table 6.5). Results for copper, zinc, iron and manganese levels in the different tissues showed no significant differences between the treatments (data not shown).

Table 6.5 Whole body and liver Se content of gilthead sea bream fed increasing contents of selenium for 42 days

Analysed dietary Se (mg kg^{-1})	0.45	0.68	0.86	1.00	1.70
Whole body Se (mg kg^{-1})	0.7 ± 0 .1	0.8 ± 0.2	1.1 ± 0.4	1.1 ± 0.4	1.2 ± 0.4
Liver Se (mg kg^{-1})	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}

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

6.4c. Gene expression

Dietary Se did not significantly affect hepatic *gpx1a* expression. However, there was a significant increase in *cat* and *gr* expression in fish fed 1.7 mg Se kg^{-1} supplementation (Table 6.6).

Table 6.6 Hepatic gene relative expression (2^-ΔΔCt) of gilthead sea bream fed increasing levels of dietary selenium for 42 days

Analysed dietary Se (mg kg^{-1})	0.45	0.68	0.86	1.00	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]

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

6.4d. Histology

Dietary Se levels did not significantly affect the occurrence of hepatic steatosis in terms of intracellular fat accumulation, broken cell margin, peripheral nucleus or sinusoid dilatation (Table 6.7). However, increase of dietary Se contents up to 1.7 mg Se kg^{-1} tended to reduce sinusoid dilatation, a morphological characteristic associated to liver damage (Table 6.7). Furthermore, only these fish presented hydropic degeneration (Table 6.7, Figure 10). No differences were found in the occurrence of melanomacrophages (Figure 9) or hyperaemia.

Table 6.7 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

Analysed dietary Se (mg kg^{-1})		0.45	0.68	0.86	1.00	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
	Sinusoids 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

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

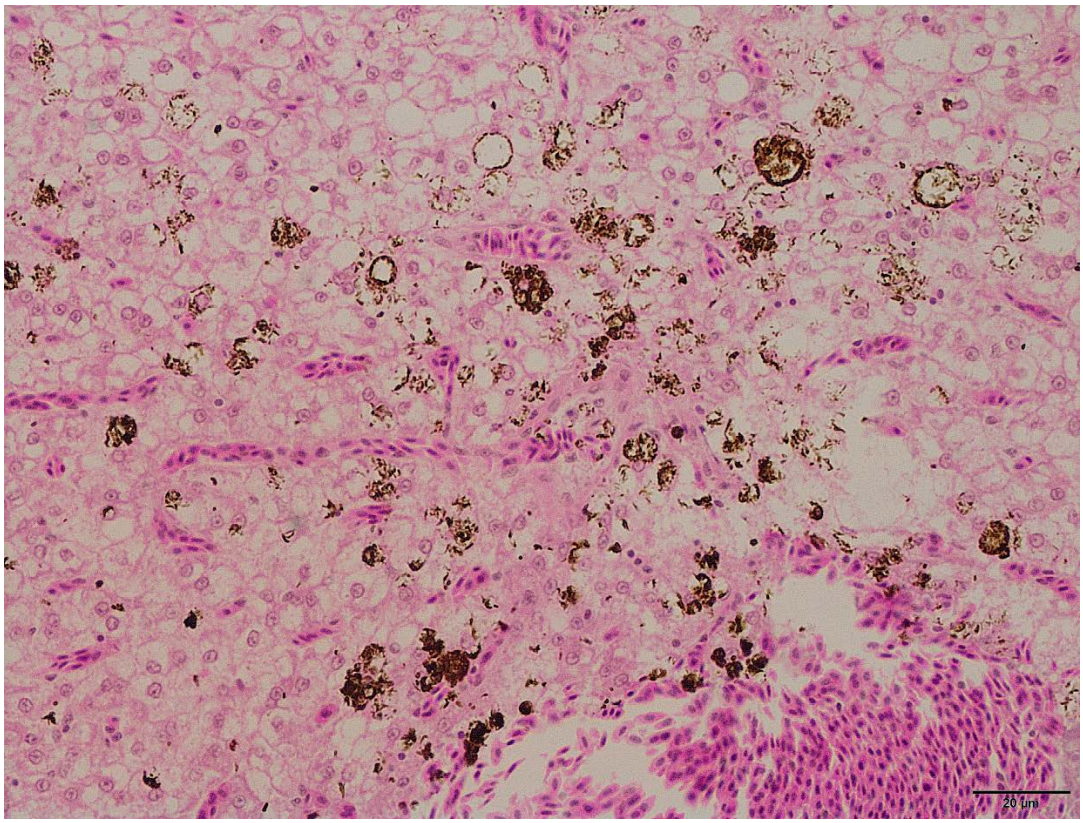

Figure 9. Microscopic view of liver (H&E) cell nucleus (40x) presenting melanomacrophages aggregates.

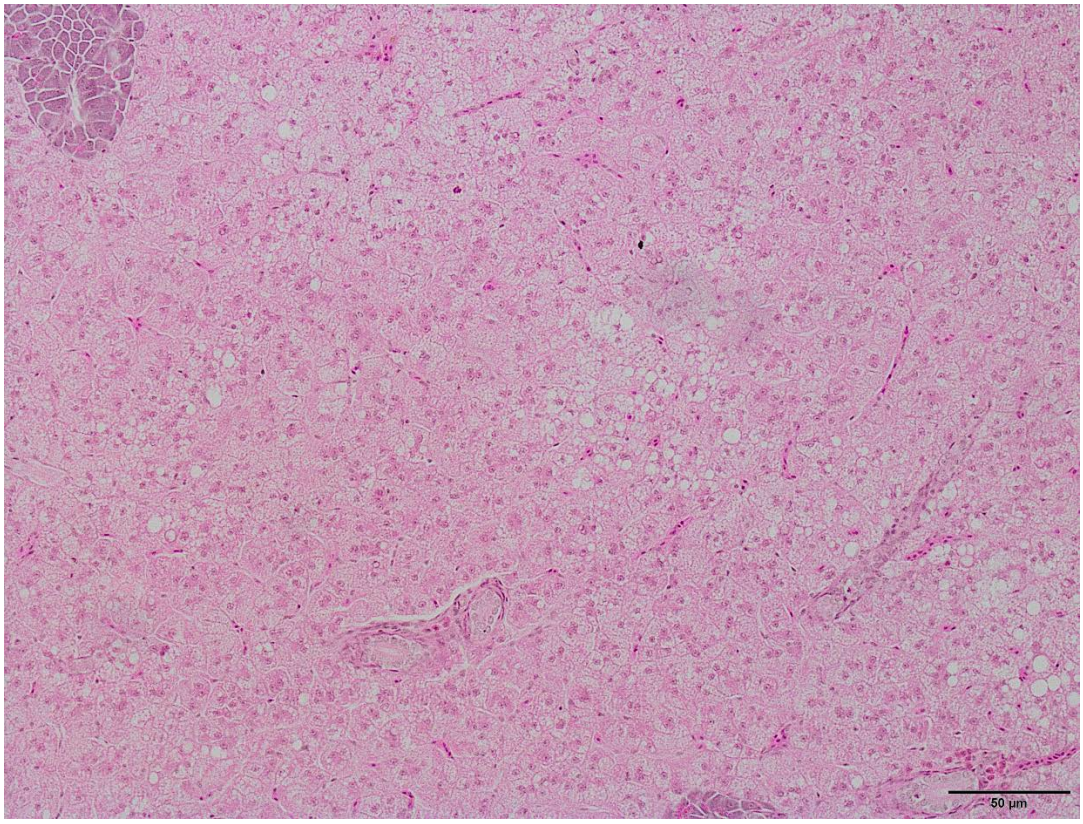

Figure 10. Microscopic view of liver (H&E) cell nucleus (20x) presenting hydropic degeneration.

6.4e. Skeletal anomalies

At the end of the trial, increase in dietary Se contents up to 1.00 mg Se kg^{-1} reduced in a 10% the total incidence of skeletal anomalies, although due to the high occurrence of these anomalies and the large deviations there were no significant differences (Table 6.8). On the contrary, further elevation of dietary Se tend to increase the prevalence of total anomalies (Table 6.8).

Table 6.8 Prevalence of skeletal anomalies in sea bream fed increasing levels of dietary Se

Analysed dietary Se (mg kg^{-1})	0.45	0.68	0.86	1.00	1.70
Fish without anomalies (%)	31±15	35±13	40±4	36±4	45±7

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

6.4f. Broken-line analyses

Broken-line results were more conservative than quadratic regressions and similar among the different parameters studied. When this analysis was applied to final standard length, body weight, SGR, whole body Se contents, incidence of skeletal anomalies, sinusoids dilation and *gpx1a* expression, the results indicated requirements of 0.91, 0.91, 0.91, 0.97, 0.93, 1.04 and 1.00 mg Se kg^{-1} diet, respectively (Figures 11, 12 ,13 and 14).

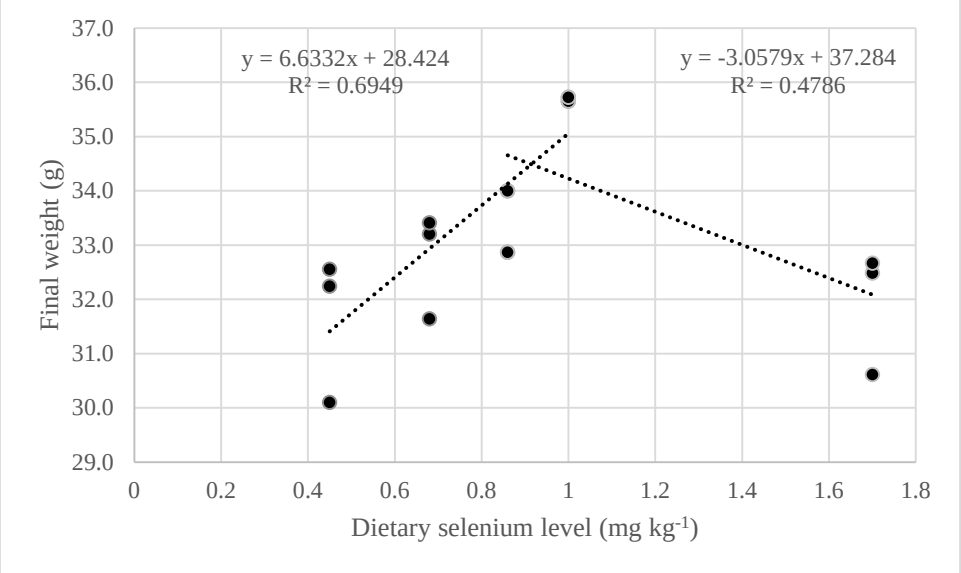

Figure 11. Relationship between dietary selenium level and final weight of gilthead sea bream fed increasing levels of dietary selenium.

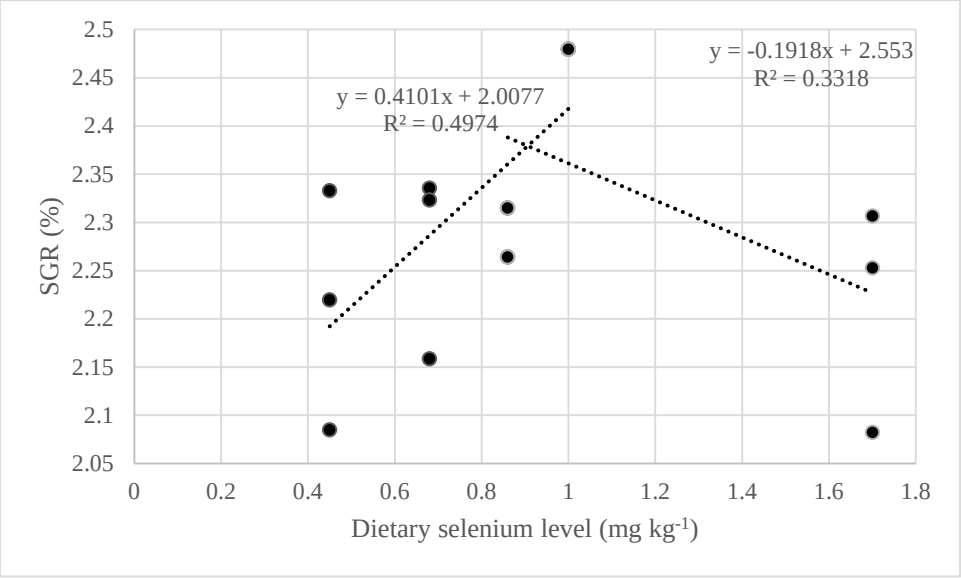

Figure 12. Relationship between dietary selenium level and SGR of gilthead sea bream fed increasing levels of dietary selenium.

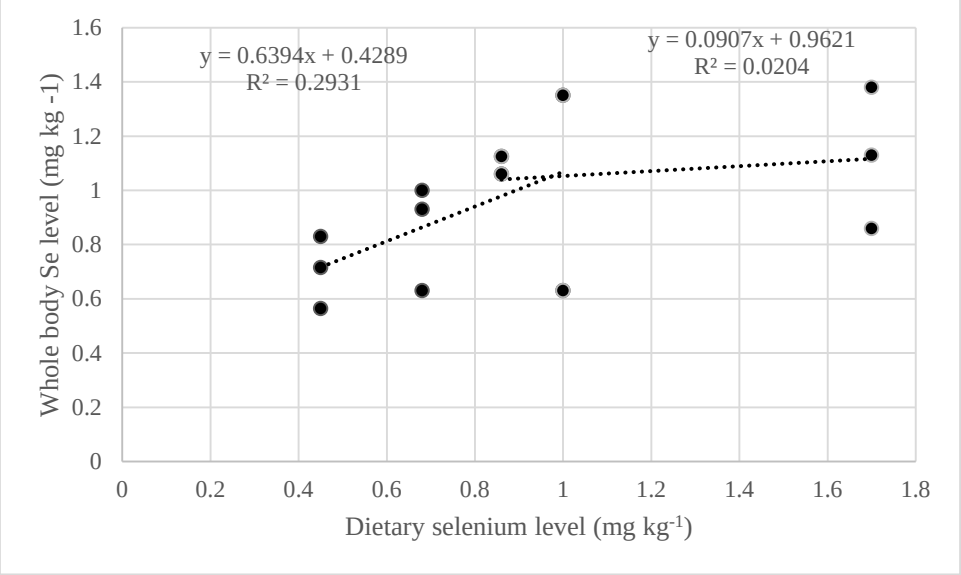

Figure 13. Relationship between dietary selenium level and whole body selenium content of gilthead sea bream fed increasing levels of dietary selenium.

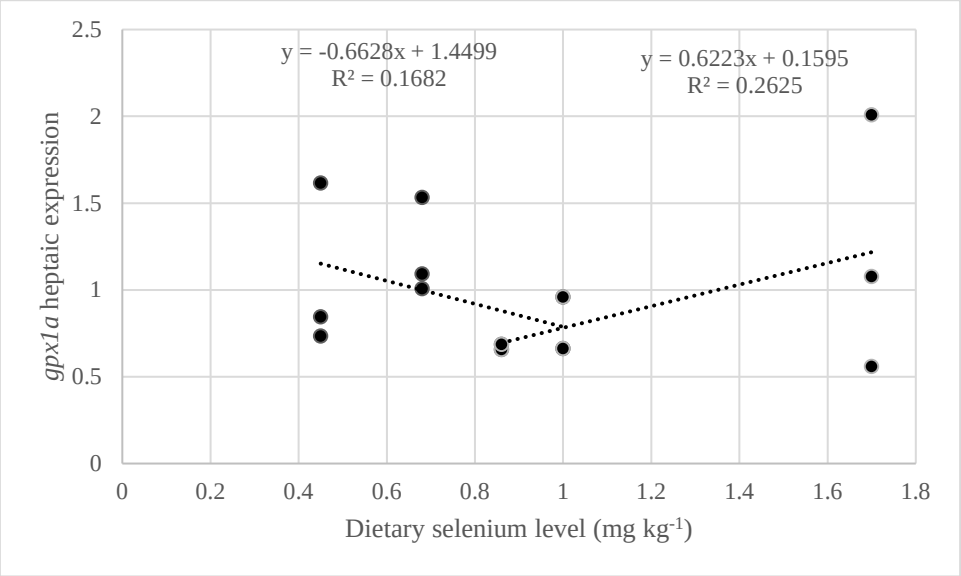

Figure 14. Relationship between dietary selenium level and hepatic *gpx1a* expression of gilthead sea bream fed increasing levels of dietary selenium.

6.5. DISCUSSION

The essentiality of Se supplementation in fish has been discussed in several reviews (Antony Jesu Prabhu *et al.*, 2016; Halver and Hardy, 2002; Khan *et al.*, 2017; NRC, 2011; Watanabe *et al.*, 1997). In the present trial, an increase in dietary Se contents up to 1.00 mg Se kg^{-1} 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, as well as in Se body contents. Indeed growth increase, in terms of final length, in fish fed up to 1 mg Se kg^{-1} was significantly related to whole body Se contents ($y = 0.76x + 10.82$, $R^2 = 0.85$). Similarly, in cobia, increasing selenomethionine dietary supplementation until 0.86 mg Se kg^{-1} increased both growth and whole body Se, reaching a plateau from this dietary level onwards (Liu *et al.*, 2010). 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.*, 2006) or high levels in juveniles (Pacitti *et al.*, 2015; Penglase *et al.*, 2014; Wang *et al.*, 2018; Zee *et al.*, 2016b) up-regulates *gpx* expression. In larval gilthead sea bream, dietary increase from 1.73 to 6.41 mg Se kg^{-1} as selenium yeast is associated to best growth and significantly down-regulates *gpx* expression, whereas further increased in Se only tends to up-regulate this gene (Saleh *et al.*, 2014). In agreement, in the present study supplementation of sodium selenite causing a dietary increase up to 0.86 mg Se kg^{-1} was correlated to a reduction in *gpx1a* expression, while further increase 1.70 mg Se kg^{-1} tended to up regulate the expression of this gene. Increase in dietary Se up to 1.00 mg Se kg^{-1} was also correlated to a reduction in skeletal anomalies. Indeed, some studies demonstrate the effect of dietary selenium levels in the regulation of the genes involved in skeletal development of gilthead sea bream larvae (Saleh *et al.*, 2014).

Se requirements in farmed fish based on growth as a criterion range from 0.12 mg Se kg^{-1} in channel catfish fed selenomethionine or yeast (Wang and Lovell, 1997) and common carp fed sodium selenite (Gaber, 2009), up to 5.39-7.37 mg Se kg^{-1} in yellowtail kingfish fed selenium yeast (Le and 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, Se body contents or expression of *gpx1a*, all suggested optimum dietary levels of around 0.94 mg Se kg^{-1} for sea bream juveniles.

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 up to 20.5 mg Se kg^{-1} (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^{-1} as juvenile rainbow trout (as sodium selenite) or chinook salmon (*Oncorhynchus tshawytscha* fed selenomethionine) (Hamilton 2004), and 11 or 15 mg Se kg^{-1} (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^{-1} resulted in a reduced growth, both in terms of standard length and body weight, comparable to those fish fed diets with the lowest levels of selenium supplementation. These results are in agreement with the reduced growth obtained because of excessive Se levels in channel catfish fed different Se sources (Wang and Lovell, 1997).

Despite the whole body Se contents in this fish were not significantly different from those of fish fed 0.86 or 1 mg Se kg^{-1}, Se deposition in liver increased linearly with increasing levels of dietary selenium. This linear increase has been described in multiple fish species including Atlantic salmon (Berntssen *et al.*, 2018), channel catfish (Wang and Lovell, 1997), cobia (Liu *et al.*, 2010), gibel carp (Han *et al.*, 2011), hybrid striped bass (Cotter *et al.*, 2007), malabar grouper (Lin, 2014), Nile tilapia (*Oreochromis niloticus*, Lee *et al.*, 2016), olive flounder (*Paralichthys olivaceus* fed selenomethionine, Lee *et al.*, 2010), rainbow trout (fed sodium selenite or selenium yeast, Hilton and Hodson, 1983; Wang *et al.*, 2018), white sturgeon (Tashjian *et al.*, 2006). Dietary Se may also affect deposition other minerals in liver, such as copper, which follows a positive correlation with Se deposition in rainbow trout (Hilton and Hodson, 1983) or Atlantic salmon (Poppe *et al.*, 1986). However, neither copper nor zinc, iron or manganese contents in liver where affected in the present study. On the contrary, elevation of selenium up to 1.70 mg Se kg^{-1} significantly up-regulated hepatic expression of *cat* denoting an increase oxidative stress associated to growth reduction in this fish. These results are in agreement with the increased catalase activity associated to 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 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).

Elevation of selenium up to 1.70 mg Se kg^{-1} in the present study also markedly up-regulated hepatic expression of glucocorticoid receptor. 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 glucocorticoid receptors (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, Se excess induces stress hormones in goldfish (Choi *et al.*, 2015) and rainbow trout (Wisseman *et al.*, 2011) or gilthead sea bream juveniles submitted to acute stress (Mechlaoui *et al.*, accepted).

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 to high levels of dietary Se in white and green sturgeon (*Acipenser medirostris*) (De Riu *et al.*, 2014). In the present study, hepatic hydropic degeneration was observed only on fish fed the diet containing 1.70 mg Se kg^{-1}, 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 1.7 mg Se kg^{-1}. 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 *et al.*, 1983) or white sturgeon (Zee *et al.*, 2016a) fed high Se dietary levels. On the contrary, dietary Se levels of 11 mg Se kg^{-1}, 10 times higher than those tested in the present study, induces a wide range of pathological alterations in liver of Atlantic salmon (Berntssen *et al.*, 2018). Despite, fish exposed to high levels of waterborne selenium show a high incidence of bone anomalies of teratogenic origin (Lemly *et al.*, 2002), in the present study only a trend to increase skeletal anomalies could be observed in fish fed 1.7 mg Se kg^{-1}.

In conclusion, the results of this study suggest that the optimum dietary levels of total selenium in diets with 10% FM with basal levels of 0.45 mg Se kg^{-1} are around 0.94 mg Se kg^{-1} to promote growth of gilthead sea bream juveniles. Moreover, feed levels of 0.85-1 mg Se kg^{-1} supplemented as sodium selenite were safe and did not negatively affect growth, catalase expression, or liver morphology. On the contrary, dietary levels of 1.7 mg Se kg^{-1} were found to be excessive and caused growth reduction, increased catalase expression and hydraulic degeneration in the liver.

CHAPTER 7: COPPER LEVELS IN DIETS HIGH IN VEGETABLE INGREDIENTS FOR GILTHEAD SEA BREAM (*Sparus aurata*) FINGERLINGS

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7.1. 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} raised n-3 HUFA contents, 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 n-3 HUFA contents in whole body 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.

7.2. 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 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 liver and whole body have also been used in Cu requirement determination studies (Antony Jesu Prabhu *et al.*, 2016 ; Lall, 2011). 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 nmetallothionein (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.

7.3. 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.

7.3a. 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.*, *submitted a*), 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 CuSO4, to contain 5.5, 7.4, 9.3, 11.0 and 32.0 mg Cu kg^{-1} diet (Table 7.1). 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 7.1 Ingredients of the experimental diets supplemented with increasing levels of Cu

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 SA	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
Analysed Cu (mg kg^{-1})	6.00	7.00	9.00	11.00	32.00

*Micronutrient premix: methionine (0.001%), lysine (1.235%), phosphate (0.67%), vitamin premix (0.18%) and mineral premix excluding Cu (0.11%), 122 mg Fe kg^{-1}, 128 mg Zn kg^{-1}, 32.2 mg Mn kg^{-1} and 0.9 mg Se kg^{-1}.

7.3b. Fish and experimental conditions

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 ± 0.4ºC, 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 (ºC)

Previous to sampling, all fish were fasted for 24 hours. 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 -80ºC until the analysis was conducted. Twenty fish per tank were sampled for radiographic assessment.

7.3c. Gene expression

i. 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.

ii. 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.

iii. Quantitative PCR

The nucleotide sequences of primers used in this study are reported in Table 7.2. 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 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 $2-\Delta\Delta Ct$ method was applied to analyse the relative changes in gene expression.

Table 7.2 Sequences of primers used for gene expression analysis

Full gene name	Gene	Nucleotide sequence (5'-3')
Beta actin	*bact*	FW: TCTGTCTGGATCGGAGGCTC
		RV: AAGCATTTGCGGTGGACG
Ribosomal protein 27a	*rpl27*	FW: ACAACTCACTGCCCCACCAT
		RV: CTTGCCTTTGCCCAGAACTT
Copper/zinc superoxide dismutase	*cuznsod*	FW: TTGGAGACCTGGGCAACGTGA
		RV: TCCTCGTTGCCTCCTTTTCCC
Catalase	*cat*	FW: ATGGTGTGGGACTTCTGGAG
		RW: AGTGGAACTTGCAGTAGAAAC
Copper transporter 1	*ctr1*	FW:CGGGTCTGCTCATCAACACCC
		RW: TGTGCGTCTCCATCAGCACCG
ATPase 7b	*atp7b*	FW: CGCTGGCCTCGTGCTTCAACC
		RW: CGACGACCGCAGGCTTCTCATTT

7.3d. Biochemical 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 600ºC 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 Burk *et al*. (1980). Approximately 20–30mg of tissue were homogenised in 1.5ml of 20% trichloroacetic acid (w/v) containing 0.05 ml of 1% butylated hydroxytoluene in methanol. Then, 2.95 ml of freshly prepared 50mM-thiobarbituric acid solution were added before mixing and heating for 10min at 100ºC. After cooling, protein precipitates were removed by centrifugation at 2000G 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.

7.3e. Histology

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-Pearson, 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.

7.3f. Skeletal anomalies

X-Ray analyses were conducted using a 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).

7.3g. Statistical analyses

All data were statistically analysed 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 $p<0.05$.

7.4. RESULTS

7.4a. Growth and productive parameters

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 7.3). Final whole body weight was significantly (P>0.05) affected by dietary Cu supplementation, where fish fed diets containing 5.5-9.3 mg Cu kg^{-1} presented the highest weight (Table 7.3). FCR and FE proved that fish fed the diet containing 11.0 mg Cu kg^{-1} presented a worse efficiency of

the feed (Table 7.3). Other productive parameters including WG, SGR, or TGC proved not significantly different between treatments (Table 7.3).

Table 7.3 Growth performance and feed utilization in gilthead sea bream fed increasing contents of Cu for 42 days

Dietary Cu (mg kg $^{-1}$)	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}

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. TGC: Thermal Growth Coefficient. FCR: Feed Conversion Ratio. FE: Feed efficiency.

7.4b. Gene expression

Results from analyses of hepatic genes revealed that dietary Cu did not have a significant effect on expression of *cuznsod.* However, there was a significant (P<0.05) 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 7.4). 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>0.05) effect of dietary Cu levels on the expression of these genes (Table 7.4).

Table 7.4 Hepatic gene expression analyses of gilthead sea bream fed increasing levels of dietary Cu for 42 days

Dietary Cu (mg kg^{-1})	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, $p<0.05$, n=3

7.4c. Biochemical analyses

Whole body Cu content was not significantly affected by dietary Cu, independently of the level of Cu supplementation (Table 7.5). Similarly, despite the slightly higher levels of Cu in liver of fish fed 9.3 and 32.0 Cu mg kg^{-1}, no significant ($p>0.05$) 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 ($p>0.05$) affected by dietary Cu supplementation (Table 7.6). Only fish fed the diet containing 9.3 mg Cu kg^{-1} showed slightly higher lipid content, but not significantly different from that of fish fed the other Cu levels. On the contrary, TBARs contents in sea bream whole body at the end of the trial were significantly ($p<0.05$) increased by the elevation of the dietary Cu levels up to 32.0 mg Cu kg^{-1} diet (Table 7.6).

Table 7.5 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, $p<0.05$, n=3.

Table 7.6 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^{-1})	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)	$155±92^{a}$	$485±143^{ab}$	$371±152^{ab}$	$252±26^{ab}$	$506±104^{b}$

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

No significant differences were found in the fatty acid composition of whole body from fish fed different Cu levels (Table 7.7). Nevertheless, increase in dietary Cu up to 9.3 mg kg^{-1} lead to the elevation in polyunsaturated fatty acids from n-6 (R^2=0.83) and, particularly, n-3 (R^2=0.62) families, and EPA/DHA (R^2=0.85) as well as an increase in ARA/EPA (R^2=0.98). However, further elevation of dietary Cu up to 35 mg kg^{-1} caused the contrary effect (Table 7.7).

Table 7.7 Fatty acid composition in whole body of gilthead sea bream fed increasing contents of Cu for 42 days (% total fatty acids)

Dietary Cu (mg kg^{-1})	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, $p<0.05$, n=3. PUFA: polyunsaturated fatty acids

7.4d. Histology

Increase in dietary Cu lead to increased liver steatosis and displacement of hepatocyte nucleus (Table 7.8, Figures 15, 16, 17 and 18). 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 (Table 7.8, Figures 15, 16, 17 and 18).

Table 7.8 Hepatic histological analyses of gilthead sea bream fed increasing levels of dietary Cu for 42 days

Dietary Cu (mg kg^{-1})	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, $p<0.05$, n=3

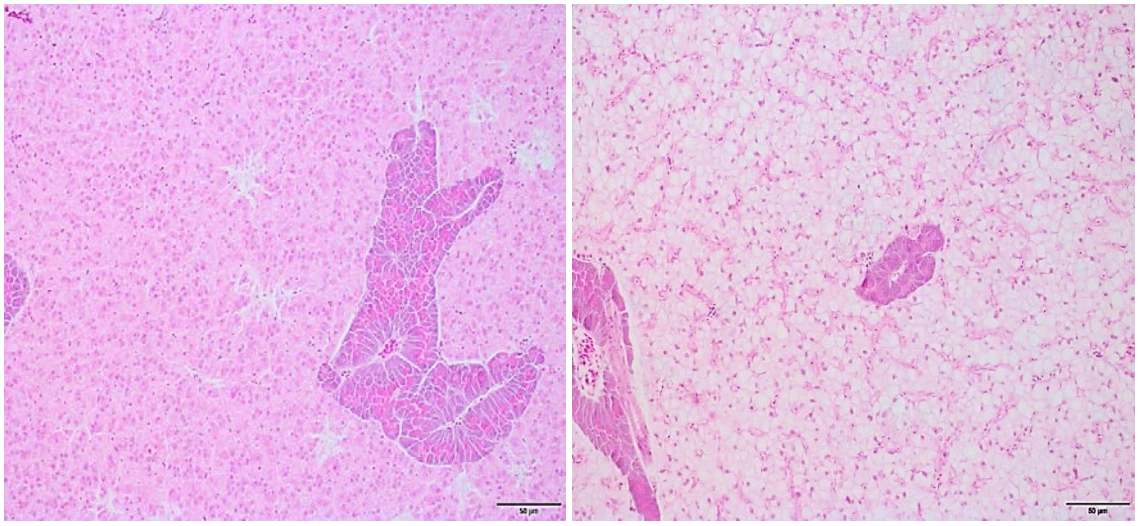

Figure 15. Microscopic view of liver steatosis (20x). Left) Low steatosis. Right) High steatosis.

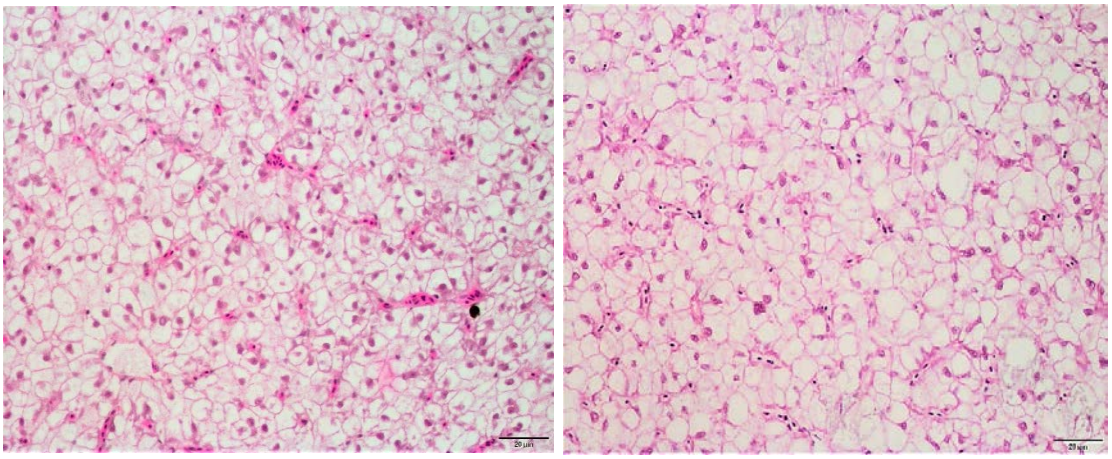

Figure 16. Microscopic view of liver cell nucleus (40x). Left) Central nucleus. Right) Peripheral nucleus.

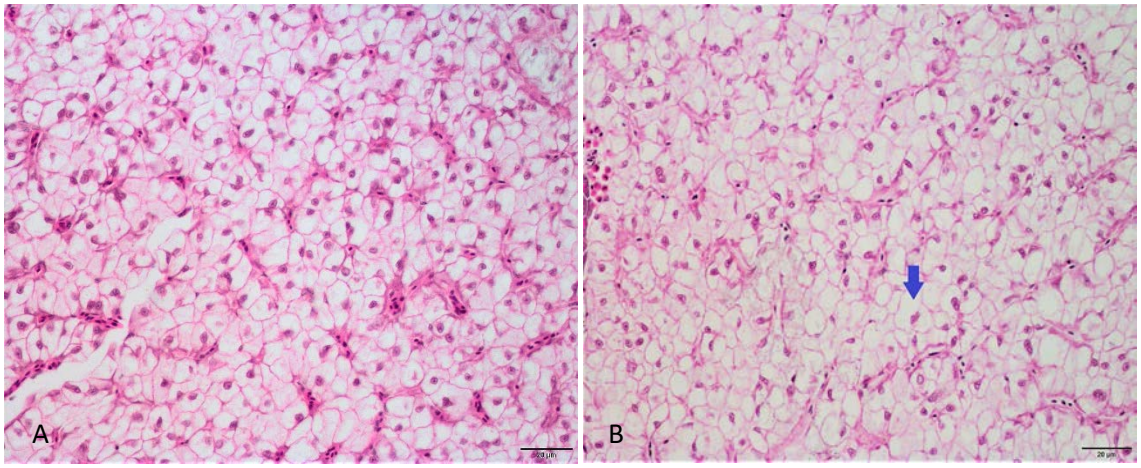

Figure 17. Microscopic view of liver cell margin (40x). Left) well-preserved cell margins. Right) Broken cell margins.

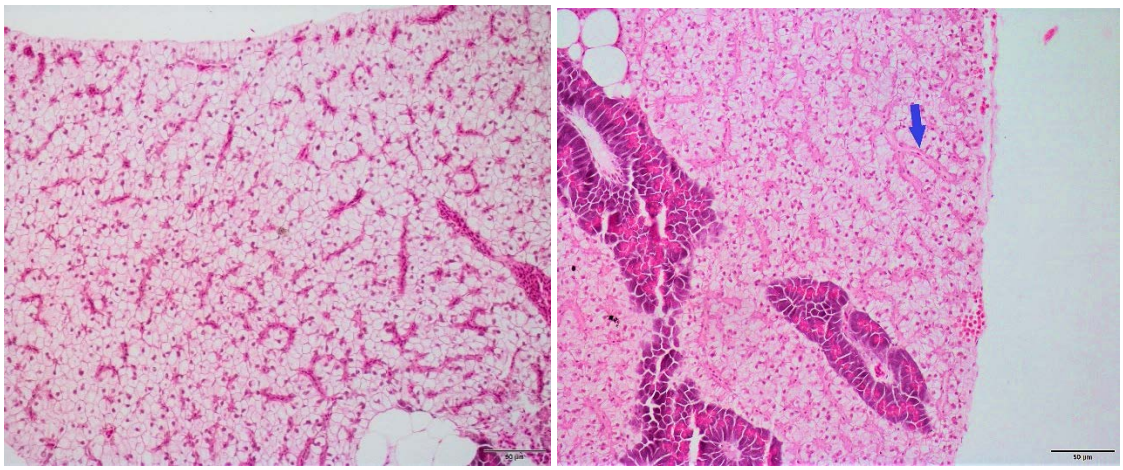

Figure 18. Microscopic view of liver cell sinusoids (20x). Left) Sinusoids with abundant erythrocytes. Right) Sinusoids dilated with plasma and without erythrocytes.

7.4e. Skeletal 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 7.9). However, fish fed the diet without Cu supplementation tended to present a higher prevalence of total skeletal anomalies.

Table 7.9 Prevalence of skeletal anomalies (%) in sea bream fed increasing levels of dietary Cu

Dietary Cu (mg kg^{-1})	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

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

7.5. 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 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 with practical, semi-purified o commercial diets (Ballester-Lozano *et al*., 2015; Benedito-Palos *et al*., 2007; 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 (Cao *et al*., 2014) or malabar grouper (Lin *et al*., 2008b) and freshwater species including channel catfish (Gatlin *et al*., 1989; Wilson and Gatlin, 1985), murrel (Abdel-Hameid *et al*., 2017), Russian sturgeon (Wang *et al*., 2016), rainbow trout (Wilson and Gatlin, 1986), common carp (Ogino and Yang, 1980), hybrid tilapia (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*., 2008b), channel catfish (1.5–5 mg Cu kg^{-1} diet, Gatlin and Wilson, 1986; Murai *et al*., 1981), 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*., 2018b). 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 with Cu requirements ranging from 6.43-9.47 mg kg^{-1} (Shao *et al*., 2010), Atlantic salmon from 8.5-13.7 mg Cu kg^{-1} (Lorentzen *et al*., 1998), tongue sole from 11-12 mg Cu kg^{-1} (Wang *et al*., 2015b) or blunt snout bream from 12-18 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 Fe or Zn (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 (Antony Jesu Prabhu *et al*., 2016; Baker, 1986; Cowey, 1992; NRC, 2011). Copper concentration in whole body or liver, as well as liver SOD activity, are among the most frequent parameters studied to determine Cu requirements (Antony Jesu Prabhu *et al*., 2016; Lall, 2011).

In the present trial, increase in dietary Cu up to 9.3 mg kg^{-1} 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^{-1}, 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 Cu kg^{-1} 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. This would mean a better protection against oxidative damage that would be reflected in the generalized increase in n-3 PUFA contents in whole body found in 9.3 mg Cu kg^{-1} diet fed fish. Indeed, increase in dietary Cu increases PUFA and reduces saturated fatty acids contents in chickens (Skrivan *et al*., 2000) or steers (Engle, 2011).

On the contrary, further increase in dietary Cu up to 11.0 and, particularly, 32.0 mg kg^{-1}, 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^{-1} diet) reduced hepatic SOD activity (Lin *et al*., 2008b), 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 Cu may initiate lipid peroxidation in biomembranes by generating peroxyl and alkoxyl radicals from the decomposition of lipid hydroperoxides (Murphy, 2001). This would explain the slight reduction in whole body PUFA contents or liver lipid levels in fish fed 32.0 mg Cu kg^{-1}. In studies with high dietary (i.e. 32.0 mg Cu kg^{-1}) or waterborne Cu levels, tissues lipid compositions are ultimately altered (Berntssen *et al*., 1999; 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^{-1} 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*., 2008b). In marine fish, excessive water borne Cu levels causing peroxidation of polyunsaturated membrane lipids or even proteins, damage cells and tissues in several organs (Isani *et al*., 2011; NRC, 2011; Roméo *et al*., 2000; Watanabe *et al*., 1997; Woody and O'Neal, 2012). In the present study, increase in dietary Cu, particularly up to 32.0 mg kg^{-1}, 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 lipoid 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^{-1} 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). The contents in other minerals were not significantly affected by dietary Cu levels, despite excessive Cu levels (100-1000 mg Cu kg^{-1} diet) 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^{-1}, 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^{-1}, 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.

8. CONCLUSIONS

1. Supplementation of vegetable meal based diets (containing 15% FM) with Zn oxide is required to promote growth in gilthead sea bream.

2. Supplementation of vegetable meal based diets (containing 15% FM) with SeMet is more effective than NaSe to reduce oxidative risk.

3. Encapsulation of inorganic minerals with chitosan to avoid their leaching reduces mineral deposition and negatively affects growth of gilthead sea bream fed vegetable meal based diets (containing 15% FM).

4. Supplementation of vegetable meal based diets (containing 15% FM) with Zn oxide, in combination with either organic or inorganic forms of Mn, is necessary to promote sea bream growth and promote whole body protein and Zn deposition of gilthead sea bream.

5. The presence of Mn in higher concentrations in plant ingredients than in animal sources suggests that practical diets based on plant ingredients (containing 10% FM) may contain sufficient Mn to cover the requirements for gilthead sea bream fingerlings.

6. A basal dietary content of 19 mg Mn kg^{-1} is sufficient to cover gilthead sea bream requirements for growth, feed utilization, tissue mineral content, whole body chemical composition, hepatic morphology or prevalence of skeletal anomalies.

7. Optimum dietary levels of Se supplementation as NaSe in diets based on plant ingredients (containing 10% FM), with basal levels of 0.45 mg Se kg^{-1}, are around

0.94 mg Se kg^{-1} to promote growth of gilthead sea bream juveniles, which is over the maximum levels allowed by European Feed Safety Authority.

8. Increase in dietary levels of Se from 1.00 mg Se kg^{-1} to 1.70 mg Se kg^{-1} by supplementation with NaSe reduces growth, increased oxidative risk and causes hydropic degeneration in the liver, indicating that these levels are already toxic for gilthead sea bream.

9. Little or even no Cu supplementation is required in gilthead sea bream fed diets based on plant ingredients (containing 10% FM), with basal levels of 5.5 mg Cu kg^{-1}.

10. Despite the maximum dietary Cu content allowed by European Feed Safety Authority is 25 mg kg^{-1}, even dietary contents of 11-32 mg Cu kg^{-1}, supplemented as Cu sulfate, negatively affected gilthead sea bream performance by reducing fish growth, increasing oxidative risk and inducing hepatic damage and cholestasis.

9. RESUMEN EN ESPAÑOL

9.1. ESTADO ACTUAL DE LA ACUICULTURA

Desde la década de 1950, la producción de pescado, tanto derivada de la pesca como de la acuicultura, ha aumentado de 20 a 171 millones de toneladas (FAO, 2018). El mayor crecimiento se ha producido en el sector de la acuicultura, alcanzando un incremento anual del 9,5% en 1990-2000. Luego de alguna reducción debido a la crisis económica mundial, el crecimiento de la producción se redujo a 5.8% para el período 2000-2016 (FAO, 2018). Hoy en día, la industria de la acuicultura produce el 53% del pescado que se consume en el mundo (FAO, 2018), mientras que las pesquerías todavía tienen la ventaja debido al rendimiento derivado del pescado no destinado a ser humano directo (FAO, 2018).

La industria de la acuicultura en la UE es predominantemente marina, con casi el 78% de la producción total, siendo dos de las tres especies de peces más cultivadas: el salmón del Atlántico (*Salmo salar*) y la dorada (*Sparus aurata*) (APROMAR, 2018). Las áreas de producción y las especies se dividen en tres ubicaciones principales: producción de agua dulce, con truchas y carpas; el Océano Atlántico Norte, con producción de salmón; y el mar Mediterráneo, con dorada y lubina europea (*Dicentrarchus labrax*).

9.2. PRODUCCIÓN DE DORADA

La dorada es un teleósteo de la familia *Sparidae* y tiene una forma comprimida que lo distingue de otros espáridos gracias a la mancha negra que cubre parcialmente el opérculo y la banda amarilla entre sus ojos. Esta especie se distribuye en todo el Mar Mediterráneo y en el Océano Atlántico desde el Reino Unido hasta Cabo Verde. Se puede encontrar en todo tipo de fondos marinos, donde se alimenta de moluscos, crustáceos y peces más

pequeños, y puede habitar en aguas salobres y estuarios, debido a su condición de eurohialina y eurotérmica (APROMAR, 2018).

El cultivo de la dorada comenzó a gran escala en la década de los 90. La producción mundial de dorada se centra en Europa y especialmente en el mar Mediterráneo, donde prácticamente todos los países la cultivan. España representa el cuarto productor mundial de dorada con 13,6 mil toneladas producidas en 2017 (APROMAR, 2018).

Para una mayor expansión de la producción de esta especie, la investigación actual se centra en la selección genética, el desarrollo de alimentos sostenibles que reemplacen la harina y el aceite de pescado (FM / FO), y la mejora de la salud de los peces mediante el uso de alimentos funcionales con altos niveles de Omega 3, vitaminas y minerales (Barazi-Yeroulanos, 2010).

9.3. NUTRICIÓN E INGREDIENTES

Los piensos en la industria de la acuicultura española representan el 31% del costo total de producción, siendo un costo importante (Salz, 2009). Tradicionalmente, los alimentos para acuicultura se producían en base a FM / FO, que son recursos limitados, altamente dependientes de la pesca y con una tendencia al alza de precios debido a la competencia con otras industrias (Tacon y Metian, 2008; Tacon y Metian, 2009; Tacon y Metian, 2015). La sustitución de FM / FO en alimentos para peces es actualmente una de las prioridades de la industria debido al aumento de los precios y la falta de estabilidad del mercado. Las harinas y aceites vegetales (VM / VO) pueden reemplazar parcialmente a FM / FO en dietas de dorada (Benedito-Palos *et al*., 2007; Gómez-Requeni *et al*., 2004; Izquierdo *et al*., 2005; Montero *et al*., 2003; Robaina *et al*., 1995), mejorando económicamente la eficiencia de la producción mientras genera un producto nutritivo y

seguro de manera sostenible. Hoy en día, es común encontrar niveles de inclusión en la dieta de ingredientes vegetales hasta un 78% del alimento total para peces herbívoros y omnívoros, y alrededor del 45% para peces marinos y carnívoros (Tacon y Metian, 2015). Los ingredientes de plantas comunes utilizados en las dietas de dorada se derivan de la soja, la colza, el girasol, el trigo, la linaza y el maíz (ARRAINA, 2015). Por lo tanto, la UE ha optado por invertir en la sustitución por ingredientes vegetales como fuentes de proteínas y lípidos.

La composición nutricional de los alimentos basados en ingredientes marinos difiere significativamente de los basados en ingredientes vegetales y puede causar efectos negativos en el crecimiento, la resistencia a las enfermedades y la productividad de los peces. Algunos de estos efectos negativos son un aumento de la deposición de lípidos en el hígado (Robaina *et al*., 1995) y un estado antioxidante reducido (Saera-Vila *et al*., 2009), incluso cuando los alimentos están formulados para contener las cantidades requeridas de aminoácidos esenciales y ácidos grasos (NRC, 2011), lo que sugiere posibles desequilibrios de micronutrientes. Esto puede deberse a los diferentes niveles de micronutrientes entre los ingredientes derivados de la pesca y los ingredientes vegetales (ARRAINA, 2015).

9.4. MINERALES EN LA NUTRICIÓN DE LOS PECES

Los minerales son elementos inorgánicos esenciales para todos los procesos de la vida en los animales, incluidos los peces. Intervienen en la formación del esqueleto, la regulación del equilibrio ácido-base y el mantenimiento de los sistemas coloidales. Muchos minerales también son componentes de enzimas, metaloproteínas y hormonas. Los minerales se pueden dividir en macrominerales, aquellos requeridos en cantidades superiores a un gramo, incluyendo calcio (Ca), magnesio (Mg), sodio, potasio y cloro; y

microminerales, requeridos en niveles más bajos, incluyendo cobre (Cu), hierro (Fe), manganeso (Mn), selenio (Se) y zinc (Zn) entre otros (Lall, 2002; Watanabe *et al*., 1997). Los peces marinos pueden obtener ciertos minerales del agua, mientras que otros deben incluirse en la dieta (Lall, 2002; NRC, 2011). Algunos de estos minerales solían incluirse en cantidades suficientes a través de ingredientes derivados del pescado, sin embargo, el aumento de la sustitución de estos por ingredientes vegetales ha alterado radicalmente el equilibrio mineral en la dieta.

Establecer requisitos nutricionales para una especie es un paso fundamental para maximizar su potencial productivo. Se han establecido varios requisitos de minerales para especies como la carpa, el bagre y los salmónidos (NRC, 2011). Sin embargo, la mayoría de los requisitos para dorada son aún desconocidos, lo que obliga a los productores de piensos a extrapolar los requisitos de otras especies para producir alimentos para doradas, algunos de los cuales pueden presentar una fisiología diferente. Por lo tanto, existe una amplia gama de nutrientes para los cuales el requisito de dorada es totalmente desconocido.

Como nutrientes esenciales, las deficiencias minerales pueden tener efectos perjudiciales en los procesos vitales que en última instancia se traducen en un deterioro de la función, lo que impide que el organismo complete su ciclo de vida (Lall, 2002). Por otro lado, el exceso de estos minerales también puede alterar los procesos fisiológicos por la toxicidad inducida, debido a la exposición tanto al agua como a la dieta. El mecanismo de acción de esta intoxicación puede variar e incluir el bloqueo de grupos enzimáticos, el desplazamiento de otro ion metálico en una biomolécula y la modificación de los sitios activos de las moléculas (Lall, 2002).

Las fuentes minerales están presentes en un amplio rango, desde los iones presentes en el agua hasta las macromoléculas, como los complejos de aminoácidos. El uso de sales

inorgánicas ha sido la principal fuente tradicional de minerales en la industria de la acuicultura. Sin embargo, la disponibilidad de algunas de estas sales puede ser baja para ciertos minerales. Algunos quelatos de aminoácidos son capaces de administrar el mineral en el sitio de absorción o directamente absorberlo como un quelato intacto, lo que reduce la pérdida del elemento (Scott *et al*., 1982).

La disponibilidad de minerales se puede reducir por interacciones con otros minerales o por la presencia de antinutrientes en el alimento (Olsen *et al*., 2007; Hansen y Hemre, 2013). Por ejemplo, ciertos complejos (factores antinutricionales) presentes en los ingredientes vegetales, como el ácido fítico, actúan como minerales quelantes en el tracto digestivo, lo que reduce su biodisponibilidad (Francis *et al*., 2001; Satoh *et al*., 2001).

OBJETIVOS

Dentro de la situación actual de la industria de la acuicultura, la tendencia es aumentar el uso de ingredientes vegetales en detrimento de ingredientes insostenibles derivados de la pesca. Este cambio trae consigo una serie de alteraciones en el perfil mineral del alimento que aún no se han entendido completamente. La nutrición mineral de dorada ha recibido poca atención a pesar de ser uno de los buques insignia de la acuicultura europea. La hipótesis de esta tesis fue que la reducción actual de FM y FO en las dietas comerciales puede afectar el equilibrio de los minerales dietéticos y sus niveles óptimos en las dietas para dorada y que el tipo de fuente mineral suplementada también puede afectar la utilización de minerales en la dieta. En un estudio preliminar que formó parte de una tesis de maestría (Domínguez, *presentado para publicación* a), se suministró una premezcla mineral completa a dorada en 6 concentraciones diferentes en dietas prácticas. Se diseñaron las dietas con altos niveles de inclusión de ingredientes vegetales y una gran variedad de parámetros específicos para cada mineral. Ese estudio proporcionó una buena aproximación de los niveles dietéticos óptimos para los diferentes minerales y, en particular, los requisitos clarificados para Zn inorgánico. Sin embargo, en ese estudio, ni los niveles óptimos para otros minerales con funciones antioxidantes como Mn, Se o Cu fueron aclarados, ni se estudió la importancia de las diferentes fuentes de minerales. Por lo tanto, la primera parte de la presente tesis tuvo como objetivo arrojar luz sobre el uso de diferentes fuentes minerales, actualmente disponibles para algunos minerales antioxidantes (Mn, Se y Zn) y su combinación en dietas prácticas con altos niveles de inclusión de ingredientes vegetales, mientras que la segunda parte consistió en una serie de estudios con varios niveles de minerales individuales destinados a comprender mejor los requisitos de Mn, Se y Cu en juveniles de dorada alimentados con dietas prácticas.

Más específicamente, los objetivos de esta tesis incluyen:

1.- Comprender el efecto de diferentes fuentes de minerales (Zn, Mn y Se) complementadas con dietas prácticas con altos niveles de ingredientes vegetales en el rendimiento del crecimiento de dorada y los indicadores seleccionados del estado nutricional de esos minerales. Para ello, se realizó un ensayo con una representación de las fuentes minerales disponibles, a saber, inorgánicos (óxidos de Mn y Zn y selenito de sodio), orgánicos (quelatos de aminoácidos de Mn y Zn y metionina de Se) y minerales encapsulados inorgánicos.

2.- Para comprender mejor los efectos específicos de las diferentes fuentes minerales para Mn y Zn, estos minerales se probaron en un ensayo con juveniles de dorada alimentados con sus fuentes inorgánicas como óxidos, sus fuentes orgánicas como quelatos de aminoácidos y combinaciones de estas fuentes.

3.- Determinar el nivel óptimo de suplementación de Mn para juveniles de dorada alimentados con dietas con altos niveles de ingredientes vegetales.

4.- Definir el efecto de la suplementación con Se en dietas prácticas para juveniles de dorada que contienen altos niveles de ingredientes vegetales.

5.- Establecer niveles seguros de suplementación de Cu en dietas para juveniles de dorada con altos niveles de ingredientes vegetales.

CONCLUSIONES

1. Se requiere la suplementación de dietas basadas en harinas vegetales (que contengan un 15% de FM) con óxido de Zn para promover el crecimiento en dorada.

2. La suplementación de dietas basadas en harinas vegetales (que contienen un 15% de FM) con SeMet es más efectiva que el NaSe para reducir el riesgo oxidativo.

3. La encapsulación de minerales inorgánicos con quitosán para evitar su lixiviación reduce la deposición de minerales y afecta negativamente al crecimiento de doradas alimentadas con dietas a base de harinas vegetales (que contienen un 15% de FM).

4. La suplementación de dietas basadas en harinas vegetales (que contienen un 15% de FM) con óxido de Zn, en combinación con formas orgánicas o inorgánicas de Mn, es necesaria para promover el crecimiento de la dorada y promover la proteína corporal total y la deposición de Zn en dorada.

5. La presencia de Mn en concentraciones más altas en los ingredientes de las plantas que en las fuentes animales sugiere que las dietas prácticas basadas en ingredientes de plantas (que contienen un 10% de FM) pueden contener suficiente Mn para cubrir los requisitos para los alevines de dorada.

6. Un contenido dietético basal de 19 mg Mn kg^{-1} es suficiente para cubrir los requisitos de crecimiento, utilización del alimento, contenido mineral en los tejidos, composición química de todo el cuerpo, morfología hepática o prevalencia de anomalías esqueléticas.

7. Los niveles óptimos de la suplementación con Se como NaSe en las dietas basadas en ingredientes vegetales (que contienen un 10% de FM), con niveles basales de 0.45 mg Se kg^{-1}, son alrededor de 0.94 mg Se kg^{-1} para promover el crecimiento de juveniles de

dorada, lo cual está por encima de los niveles máximos permitidos por la Autoridad Europea de Seguridad Alimentaria.

8. El aumento en los niveles dietéticos de Se de 1.00 mg Se kg^{-1} a 1.70 mg Se kg^{-1} mediante la suplementación con NaSe reduce el crecimiento, aumenta el riesgo oxidativo y causa la degeneración hidrópica en el hígado, lo que indica que estos niveles ya son tóxicos para la dorada.

9. Se requiere poca o incluso ninguna suplementación de Cu en las dietas a base de ingredientes vegetales (que contienen un 10% de FM), con niveles basales de 5,5 mg de Cu kg^{-1} para dorada.

10. A pesar de que el contenido máximo de Cu en la dieta permitido por la Autoridad Europea de Seguridad Alimentaria es de 25 mg kg^{-1}, incluso el contenido dietético de 11-32 mg de Cu kg^{-1}, suplementado como sulfato de Cu, afectó negativamente el rendimiento de la dorada al reducir el crecimiento de los peces, aumentando el riesgo oxidativo e induciendo daño hepático y colestasis.

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