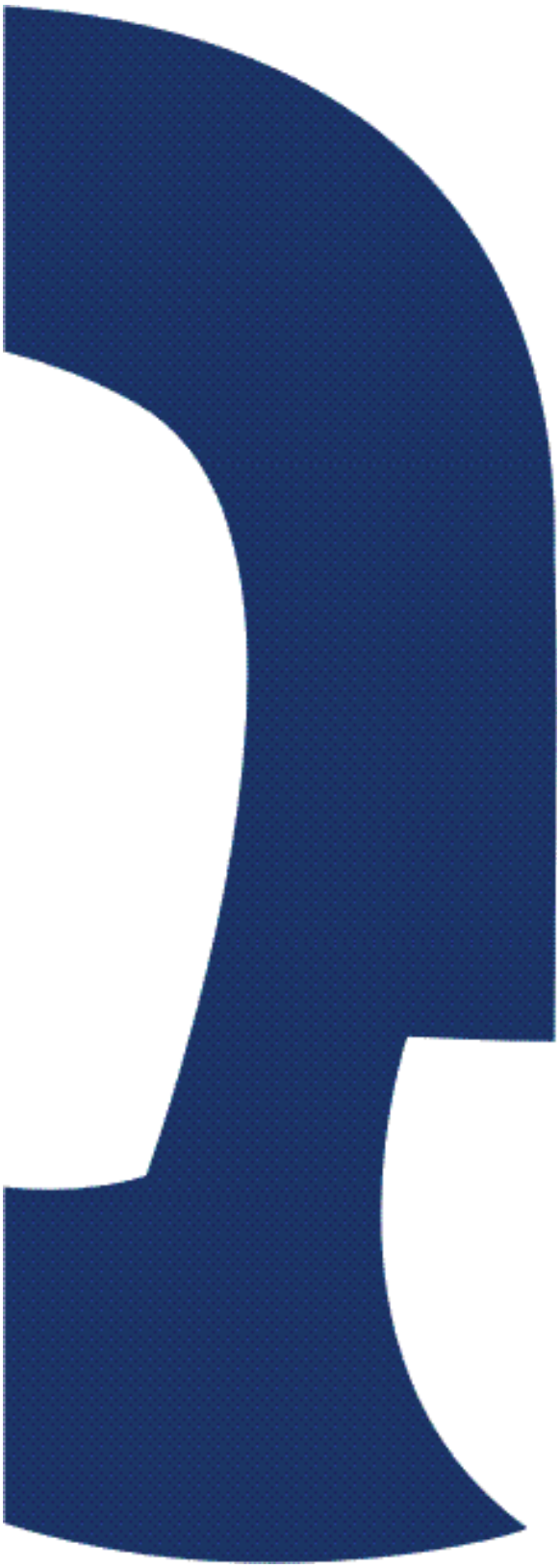

FACULTAD DE CIENCIAS DEL MAR

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

EFFECTS OF DIETARY COPPER IN GILTHEAD SEABREAM FINGERLINGS (*Sparus aurata*)

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Effects of dietary copper in gilthead seabream fingerlings (*Sparus aurata*)

Trabajo vinculado al proyecto PerformFISH (Integrating Innovative Approaches for Competitive and Sustainable Performance across the Mediterranean Aquaculture Value Chain), financiado por la Comisión Europea (EC) a través del programa Horizon 2020 (Societal Challenges).

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Table of contents

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Gilthead seabream (*Sparus aurata*) is the third most produced fish in aquaculture of EU. In commercial feeds for this species, marine ingredients (fish meal (FM) and fish oil (FO)) are being increasingly substituted with ingredients of terrestrial origin with different mineral profiles. Copper (Cu) is an essential trace element necessary for the correct functioning of numerous Cu-dependent enzymes. Fish can uptake Cu from the water or diet, and it is particularly accumulated in eyes, liver, heart and brain. Besides, high concentrations of Cu may exert toxic effects. In general, ingredients of terrestrial origin have higher Cu levels than marine ingredients. With the changing ingredient profile of commercial aquafeeds, it is necessary to define the optimal dietary supply of Cu to ensure better performance, and to avoid excess supplementation. Thus, the aim of this study was to evaluate optimal dietary inclusion level of Cu in low FM-FO diets for gilthead seabream fingerlings.

A basal diet closely mirroring practical seabream feeds was formulated with low inclusion of FM (10%) and FO (6%). Five vegetable-based diets contained a mineral premix of $CuSO_4$ in five different concentrations at 6, 7, 9, 13 and 35 mg Cu/kg diet were fed to seabream fingerlings (12.6 ± 1.4 g, mean ± SD). Fish 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 lowest dietary Cu levels tested (6 mg Cu/kg) did not caused low growth or other signs of Cu deficiency. Therefore, the lowest dietary Cu levels tested in this study were not enough to observe clear Cu deficiency symptoms and this level would be enough to meet the needs for correct growth of seabream fingerlings. Nevertheless, dietary increase from 6 to 9 mg Cu/kg, lead to a generalized increase in n-3 HUFA and slight improvements in productive parameters such as weight gain, feed efficiency, specific growth rate and thermal growth coefficient, as well as an up-regulation of *cat* gene expression. These results suggest that a maximum inclusion of 9 mg Cu/kg would be recommended to maximize n-3 HUFA deposition in seabream. Moreover, increase in dietary Cu levels over 9 mg/kg Cu led to reduction in HUFA levels, down-regulation of *cat* expression and, most importantly, an increase in liver damage. The hepatocyte damage could be seen in the histological results through an increase of steatosis, peripheral nucleus, break of the cellular margin and the dilatation of sinusoids. All these effects denote clear symptoms of a Cu excess in seabream fingerlings when dietary Cu is raised over 9 mg/kg Cu.

Keywords: aquaculture, gilthead seabream, Cu, micronutrient, diet, requirement.

INTRODUCTION

1. Updated situation of world aquaculture and gilthead seabream production

In 2015, world aquaculture production reached 106 million tons. In the European Union the production was of 1.3 million tons, being Spain the member state of the European Union with a greatest harvest, reaching 289,821 tons that year (APROMAR, 2017).

Within the aquaculture fish production, it is estimated that the total production of gilthead seabream (*Sparus aurata*) for the year 2015 was 158,389 tons (Fig. 1). However, in more recent years like 2016, the production of seabream in Europe and the rest of the Mediterranean is estimated at 195,853 tonnes. For 2017, an additional growth of 5.3% is estimated, reaching 206,000 tons.
In Spain the production was 13,740 tonnes, being seabream the third most produced species (16,231 t) in 2015. Within Spain, the Canary Islands is the third region in seabream production.
The total value of the 13,740 tons of Spanish seabream sold was 79.4 million euros, because the average price in the first sale of aquaculture seabream produced in Spain in 2016 was 5.78 euros/kg (APROMAR, 2017).

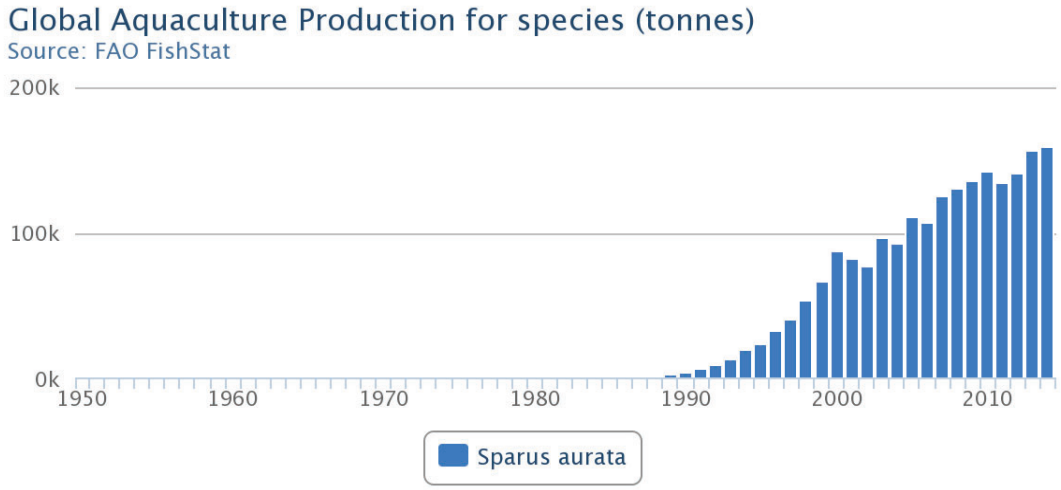

Figure 1. Global aquaculture production of gilthead seabream (FAO, 2015)

Sparus aurata commonly known as gilthead seabream (or "dorada" in Spain), is a teleost from the *Sparidae* family. Gilthead seabream has an oval body, rather deep and compressed. The overall colour of the body is silvery grey, with characteristic spots like a large black blotch at the origin of the lateral line extending on the upper margin of the operculum, a golden frontal band between eyes, also the fork and tips of the caudal fin are black (Fig. 2). Gilthead sea bream is commonly found throughout the Mediterranean and the Atlantic Ocean including Canary Islands. This is a euryhaline and benthopelagic species found in coastal environments (≈ 30 m), but adults may be found up to 150 m deep. Its diet is mainly carnivorous (mollusc, crustaceans and fish), but accessorily herbivorous. Also, this species is a protandrous hermaphrodite, is born as male and then turns into female (Pavlidis et al., 2011).

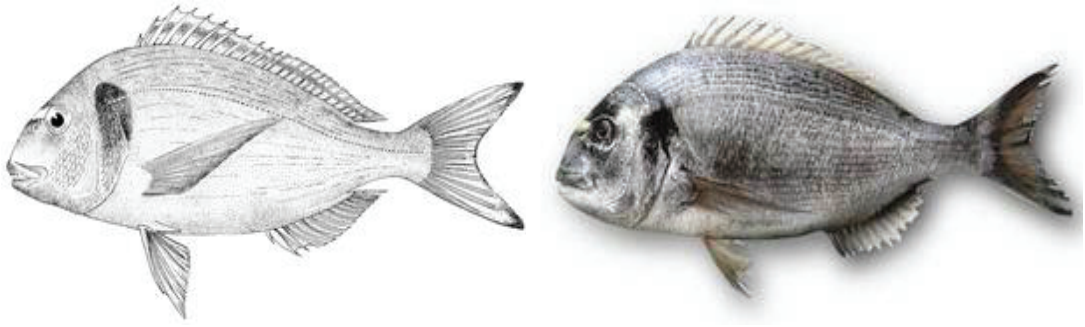

Figure 2. Gilthead seabream (Sparus aurata) (FAO, 2018)

One of the challenges humanity is facing is to feed the 9.6 billion people who will inhabit the Earth in the year 2050. This challenge is complex due to the limited availability of natural resources, the need to respect ecosystems and the 800 million people suffering chronic malnutrition, thus it is necessary to increase the agricultural production in a sustainable way.

Fishing has reached the maximum of sustainable exploitation of wild fishery resources. The stabilization of fisheries, with the increase of the demand of aquatic products, has impelled the development of the aquaculture, producing 106 million tons in 2015, and surpassing the production of the fishing in 12.3 million tons (Fig. 3) (APROMAR, 2017).

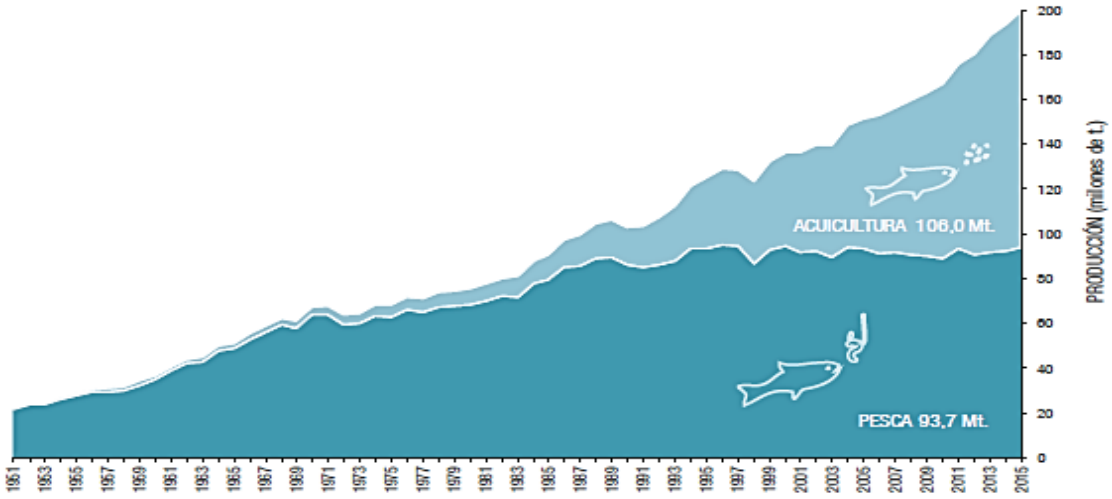

Figure 3. Evolution of world aquatic production (aquaculture and fishing) in the period 1950-2015 (FAO, 2018)

Of all the products of aquatic origin, the proportion aimed to direct human consumption has gone from 67% in 1960 to over 87% in 2014. The rest is mainly used as raw material for animal feed, including aquaculture (APROMAR, 2017). The main products used for aquaculture feed were fishmeal (FM) and fish oil (FO). This is a problem because to reach a 100% sustainable aquaculture, limited resources of aquatic origin should not be used in diets. For that reason, aquaculture is struggling today to solve this problem, trying to replace as much FM and FO as possible by vegetable meals (VM) and vegetable oils (VO). Thus, complete control of the production cycle from the beginning to the end requires the use of alternative ingredients to reduce the dependence of aquaculture on fisheries. Different alternative ingredients are being used to replace FM in diets for gilthead seabream. Despite FM is mostly a source of protein and amino acids, it also contributes to fish diets with other essential nutrients such as minerals and vitamins. Copper is one of those minerals.

2. Importance of dietary Cu for fish

Copper (Cu) is an essential trace element for all animals including fish. It is a vital component of several enzymes that are involved in oxidation–reduction reactions and occurs tightly bound to proteins as metalloenzymes. These metalloenzymes take part in cellular energy production, neurotransmission, collagen synthesis, melanin production and protection of cells from free radical damage (through CuZn superoxide dismutase) (Halver and Hardy, 2002).

Cu concentration is high in eyes, liver, brain and heart (Watanabe *et al.*, 1997; Halver and Hardy, 2002). A deficiency or excess of copper can have negative consequences for fish health. For example, at high concentrations Cu can be toxic, because it is an accumulative contaminant heavy metal (Tacon, 1992). Cu toxicity may cause damage to gills, necrosis in liver and kidney, and reduce growth, Cu tissue levels and feed efficiency (Tang *et al.*, 2013;Watanabe *et al.*, 1997). Otherwise, the effects due to a Cu deficiency can be a reduction of growth, occurrences of cataracts, and also affects the activities of cytochrome c oxidase in heart and copper-zinc superoxide dismutase (CuZnSOD) in liver (Watanabe *et al.*, 1997; Tacon, 1992; Wilson & Gatlin, 1986; Halver & Hardy, 2002). If a Cu deficiency affects the activity of the CuZnSOD enzyme, this can affect all the tissues studied. This is because the enzymes superoxide dismutase (SOD) and catalase (CAT) act as antioxidants by intercepting and inactivating oxygen free radicals in the cells. The SOD is the first that intervenes and prevents the initialization of the chain reaction of the radicals produced by the superoxide anion, because the superoxide ($O_2\cdot$) is dismuted to hydrogen peroxide (H_2O_2). Afterwards, CAT reduces the peroxide (H_2O_2) to water (H_2O) (Betancor, 2012; Winston and Di Giulio, 1991). The disturbances in Cu metabolism are secondary effects of hepato-biliary disfunction in relation to cholesterol and bile metabolism when fed plant-ingredient based diets (Antony Jesu Prabhu, 2015).

Copper can be present in the diets based in FMFO or VMVO, but there are different micronutrient contents in fish or vegetable ingredients (ARRAINA, 2015). Besides, the presence of anti-nutrients present in VMVO (Hansen and Hemre, 2013) that act chelating minerals in the digestive tract, reducing its bioavailability (Francis *et al.*, 2001; Satoh *et al.,* 2001). In addition, it has been observed that FMFO-based diets have more Cu than those based on VMVO (ARRAINA, 2015; Antony Jesu Prabhu, 2015).

Cu may be present in feeds as a supplement in organic and inorganic form. The organic forms used can be metal amino acid complexes, metal amino acid chelates and metal proteinate, while the inorganic forms are copper sulfates ($CuSO_4$), oxides (CuO) and chlorides ($CuCl_2$). The most used form of inorganic source is the copper sulfate ($CuSO_4*5H_2O$ in diet), some authors have observed that the addition of organic copper shows a better bioavailability and efficiency than inorganic forms (Dobrzanski *et al.*, 2008; Shao *et al.*, 2010; Antony Jesu Prabhu *et al.*, 2014). On the other hand, some studies indicate that inorganic sources are equally efficient or even better than chelated sources of copper (Apines *et al.*, 2003).

Among the different markers chosen to determine copper requirements the most frequently used are: Cu concentration in whole body, liver, vertebrae, serum and plasma, in addition to the activity of hepatic CuZnSOD and weight gain (Antony Jesu Prabhu *et al.*, 2014). This is because it has been observed that liver Cu concentration is positively correlated to dietary Cu concentration, and whole-body Cu concentration is influenced by liver Cu concentration (Lorentzen, *et al.*, 1998; Antony Jesu Prabhu *et al.*, 2014). Therefore, an excess of dietary copper produces oxidative stress, which affects negatively the cells of the tissues studied, and this causes an increase in SOD activity to counteract the effects of oxidative stress. Otherwise *cat* can be measured by the method involving the reaction of hydroperoxide (H_2O_2) reduction. The absence of *cat* in blood can influence lipid peroxidation reactions. High correlations are found between the activity of SOD and CAT, and the activity of SOD and CAT with peroxidase (Rudneva, 1997). Thus, more recently, molecular markers for related enzymes are also used such as *CuZnsod* and *cat* genes expression.

Besides, other markers as weight gain are not considered as good biomarkers as mineral contents, since clinical deficiencies are preceded by alterations in normal tissue or mineral levels throughout the body in fish (Shearer, 1984). In addition, in tissues such as blood, Cu is not used because it passes quickly from the intestine to the liver and the window of detection is very short.

Thus, Cu is an essential nutrient whose dietary levels can easily lead to deficient or excess problems, particularly when FM is replaced by VM in aquafeeds. Due to the need to reduce the ingredients of marine origin (including FM) in aquafeeds to increase their environmental and economic sustainability and reduce the capture pressure in fisheries resources, FM must be replaced by VM. Since Cu levels and availability may be reduced by the FM replacement by VM, dietary Cu supplementation should be studied. However, the optimum Cu levels in diets for gilthead seabream have not been defined yet. Therefore, the main objective of this study was to determine the effect of dietary Cu levels in seabream growth performance and health, including tissue biochemical composition, gene expression and histology.

MATERIAL AND METHODS

1. Feeding trial

Four hundred and fifty gilthead seabream (*Sparus aurata*) juveniles were randomly distributed in fifteen 500 L fiberglass tanks. Water intake was placed at the top of the tanks, close to the edge and oriented to produce a circular water flow, whilst water outlet was situated at the bottom of the tank, allowing for flushing and feed recovery at the end of each day. Fish average initial weight was 12.6 ± 1.4 g (mean ± S.D.). Fish were manually fed until apparent satiation four times per day. Each diet was tested in triplicates during 42 days, when fish body weight triplicated the initial weight. The trial was carried out in the facilities of the Aquaculture Research Group (GIA) of the EcoAQUA Institute in University of Las Palmas de Gran Canaria, Spain. Seawater temperature and water-dissolved oxygen were daily recorded (22.9 ± 2.3 ºC, mean ± SD). Fish were kept under a natural photoperiod of approximately 12 h light.

Five different isoenergetic and isonitrogenous practical diets were designed based on vegetable ingredients and with low levels of marine ingredients (10% FM and 6% FO). They contained 5 different levels of Cu based on NRC (2011) requirements for other species (6, 7, 9, 13 and 35 mg/kg). Diets were manufactured by Skretting Aquaculture Research Center (Stavanger, Norway) in the frame of the PerformFISH project. At days 0, 18 and 42 of the trial, growth was measured based on standard length (cm) and weight (g). All the fish were fasted for 24h previous to the sampling. During samplings fish were caught and introduced into an anaesthetic tank containing clove oil (Guinama S.L.U., Valencia, Spain) to reduce stress and improve handling. The fish that were meant to be returned to the tanks were put in a recovery 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.

The productive parameters are a numerical quantity and serve to summarize and give information about the population data of a study. The calculation of the productive parameters is conducted through stablished formulae. At the end of the trial productive parameters were calculated including Weight gain **(**WG), Food Efficiency (FE), Feed Conversion Ratio (FCR), Survival Rate, Specific Growth Rate (SGR), and Thermal Growth Coefficient (TGC) using the following formulae:

- Weight gain **WG** = [(final body weight **–** initial body weight) / initial body weight] × 100.

- Food Efficiency **FE** = [(final body weight **–** initial body weight) **/** food intake]

- Feed conversion ratio **FCR** = Ingested food / biomass generated

- **Survival (%)** = (final fish number **/** initial fish number) × 100.

- Specific growth rate **SGR (%)** = (Ln W1 – Ln W0) / days × 100

- Thermal growth coefficient **TGC:** $(W1^{1/3} – W0^{1/3})$ / days × T

Where **W0:** initial body weight (g), **W1:** final body weight, **T:** Temperature (°C)

3. Proximate and fatty acid composition

In the biochemistry laboratory samples of homogenized whole fish of seabream were analysed, to determine the proximate composition and fatty acids methyl esters (FAMES). These samples were stored in a freezer at -80ºC before the analysis began.

3.1. Total crude proteins quantification

Proteins were calculated based on the total nitrogen composition of the samples through the technique described by Kjeldhal (AOAC, 1995). The method consists in the digestion of the samples with concentrated sulphuric acid at 400ºC in presence of a copper catalyser, followed by a distillation. The method is divided into three steps:

1) Digestion of the simple with concentrated sulphuric acid (H_2SO_4) in presence of a Cu-Se catalyser to convert all the nitrogen into ammonium sulphate.

Organic $N_2 + H_2SO_4 \rightarrow CO_2 + H_2O + (NH_4)_2SO_4$

2) Liberation of ammonia (NH_3) from the digested sample by addition of sodium hydroxide in excess and vapour distillation of this ammonia in boric acid 1%.

$(NH_4)_2SO_4 + NaOH + Q \rightarrow NH_3 + HBO_2 \leftrightarrow NH_4 \cdot + BO_2 \cdot$

3) Determination of the liberated ammonia and titration with standard hydrochloric acid (HCl).

$BO_2 \cdot + H^+ \leftrightarrow HBO_2$

Finally, the protein content has been calculated using the following formula:

$$\% \text{ protein} = \frac{(\text{sample HCl ml} - \text{white sample HCl ml}) * 0.1 * 14.007 * 6.25 * 100}{\text{sample weight (mg)}}$$

3.2. Total crude lipids quantification

The analytical method used to determine total crude lipids in the simple was described by Folch *et al*., (1957) using a mixture of chloroform-methanol (2:1) containing 0.01% (Butylated hydroxytoluene) BHT. The solvent was then evaporated by using 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:

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

3.3. Moisture quantification

Moisture is calculated by drying the sample in an oven at 110ºC until constant weight (AOAC, 1995). Then, the moisture content is calculated as:

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

3.4. Ash quantification

Ash content is determined by incineration of the simple using a muzzle furnace at 600ºC until constant weight (AOAC, 1995). The ash content is then calculated by the formula:

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

3.5. Transesterification in acid medium (methylation)

For fatty acids methyl esters (FAMES) determination, total lipids are trans-esterified in sulfuric acid (1%) and methanol according to Christie (1982) methodology. FAMES are diluted in hexane and its 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 acids/100g total fatty acids.

4. Histology

Five fish per tank were sampled for histological analysis of the seabream liver at the end of the trial. Tissues were stored in 10% buffered formaldehyde. Steatosis, broken cell margin, peripheral nucleus and sinusoids dilatation in liver were observed in the histological analysis. To do it, first, liver samples must go through several processes. Firstly, the samples were further segmented to allow a better penetration of the reagents and introduced in histology cassettes. Afterwards, the cassettes were put into a tissue processor (Fig. 4) to embed the samples in paraffin, where they go through different alcohols with gradually increasing grades beginning with 70º and ending with 100º, being the last two steps xylene and paraffin, this process was done for about 24 h.

Figure 4. Processor (Leica, Nussloch, Germany)

After this, the samples were passed to a metal mould plate with paraffin until it solidifies. Once the paraffin block was obtained it was first carved and then trimmed 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. Next, samples were stoved for 30 min at 100 °C. The samples were then stained with haematoxylin-eosin staining (Martoja and Martoja-Pierson, 1970). Once the slides were prepared, the liver steatosis, peripheral nucleus location, breakage of cell margins and dilatation of sinusoids (Fig. 5-8) were evaluated in a 0-3 scale, where 0 was absence and 3 was the presence in most of the tissue.

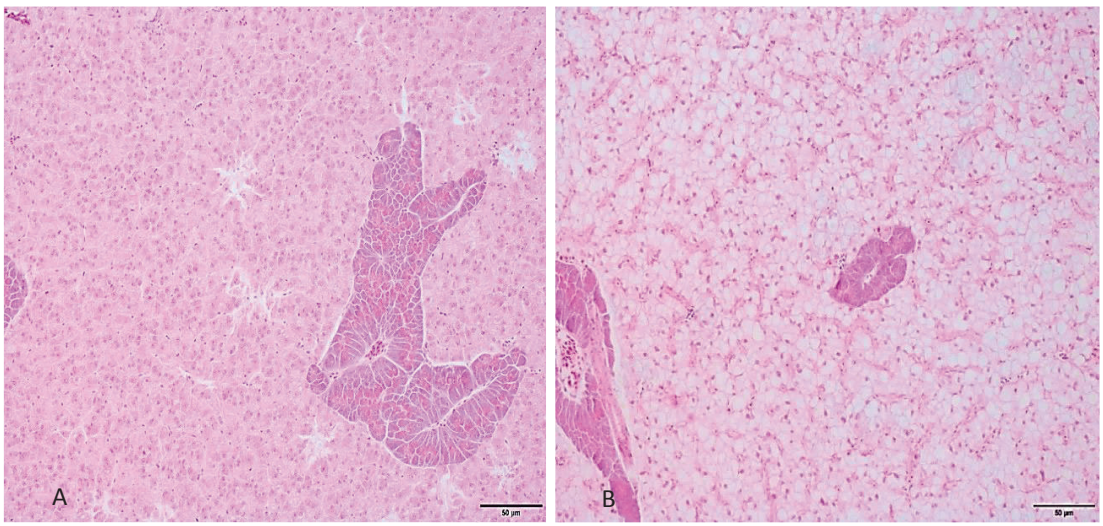

Figure 5. Microscopic view of liver steatosis (20x). A) Low steatosis. B) High steatosis

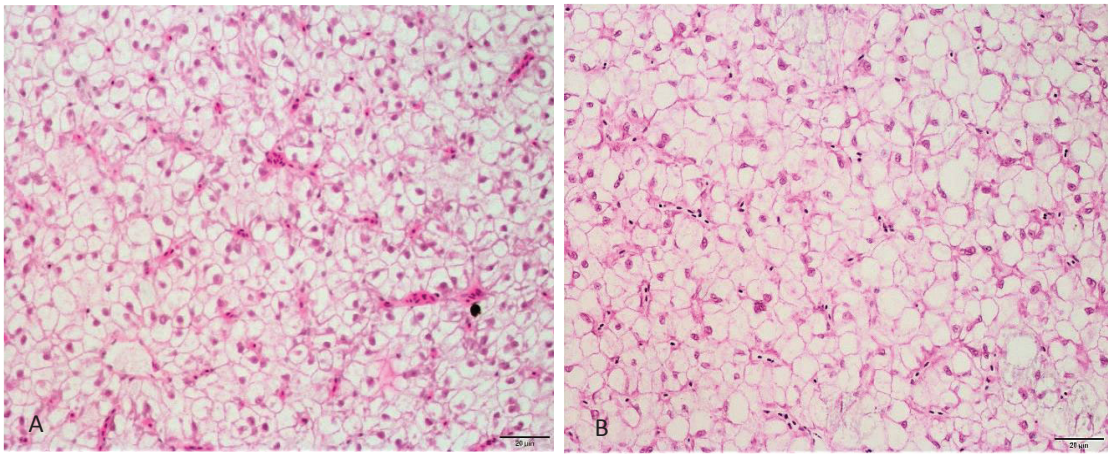

Figure 6. Microscopic view of liver cell nucleus (40x). A) Central nucleus. B) Peripheral nucleus

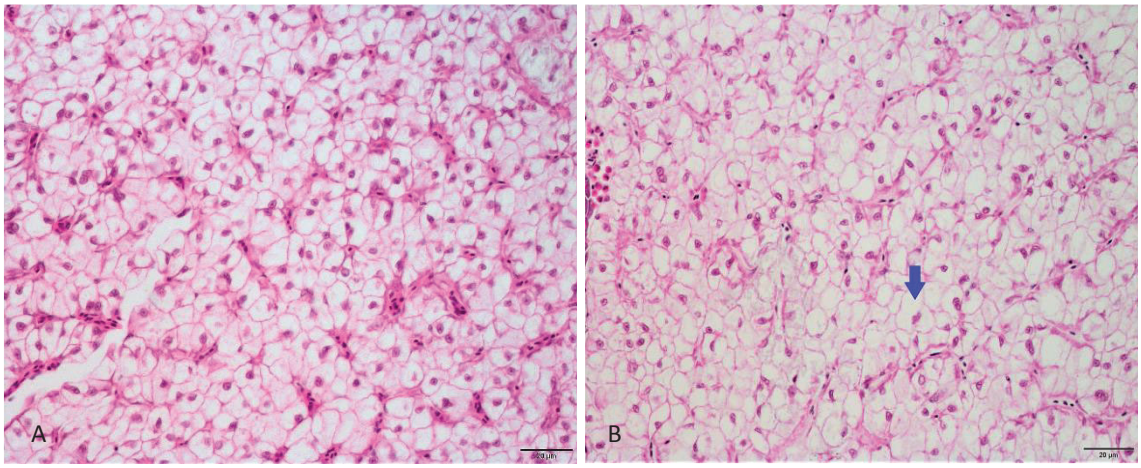

Figure 7. Microscopic view of liver cell margin (40x). A) well-preserved cell margins. B) Broken cell margins

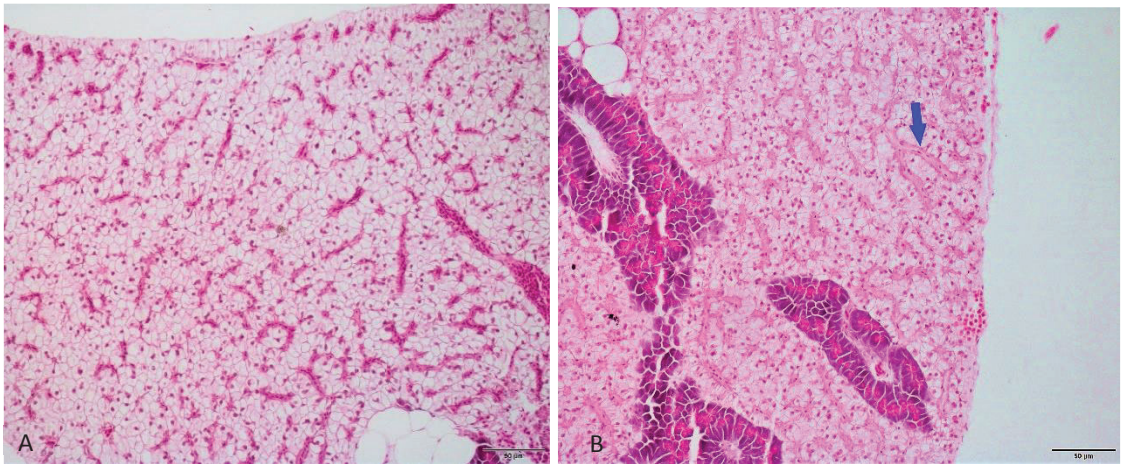

Figure 8. Microscopic view of liver cell sinusoids (20x). A) Sinusoids with abundant erythrocytes. B) Sinusoids dilated with plasma and without erythrocytes

5. Gene expression

To analyze the gene expression, three livers were taken from three different fish per tank. These samples were stored at -80 ºC.

5.1. RNA extraction

The RNA was extracted from the liver samples with a kit-RNeasy. Before carrying out the extraction, a pool was done with the three livers of each tank, so finally there are 15 pools, one for each tank. For the extraction process, 60-80 mg of the pool tissue are taken and placed in an eppendorf. Then, 1 ml of trireagent® and pellets are added to homogenize the sample in the homogenizer (tissuelyser II, Quiagen). Next, 200 µl of chloroform are added and the samples are centrifuged 15 minutes at 12000 g and 4 ºC. After centrifuging, three phases are separated in the eppendorf, above the RNA, then the DNA and underneath the reagents and proteins. After that, the RNA is transferred to another eppendorf, to which ethanol and different buffers (RW1 and RPE) are added, always passing through a filtering column, centrifuging and discarding the filtered liquid. Finally, water free of RNAses is added following the same procedure as before.

5.2. RNA quality check

Once the RNA was extracted, the quality and quantity of RNA was assessed.

The integrity and relative quantity of RNA was assessed by electrophoresis. To carry out the electrophoresis a 1.4% agarose gel is made. Before putting the RNA sample into the gel, it is denatured by temperature (10 minutes at 65ºC) in a thermal cycler (Fig. 9 B). Then, the gel is placed in an electrophoresis cuvette (Fig. 9 A). In the first well of the gel the marker ʎ125 (6 µl) is placed and in the rest of wells, a mixture of the RNA sample (5 µl) and a 6X buffer (1 µl). Finally, the gel starts running, first 1 minute at 200 volts and then 1 hour at 60 volts. The image of the result is seen on a computer.

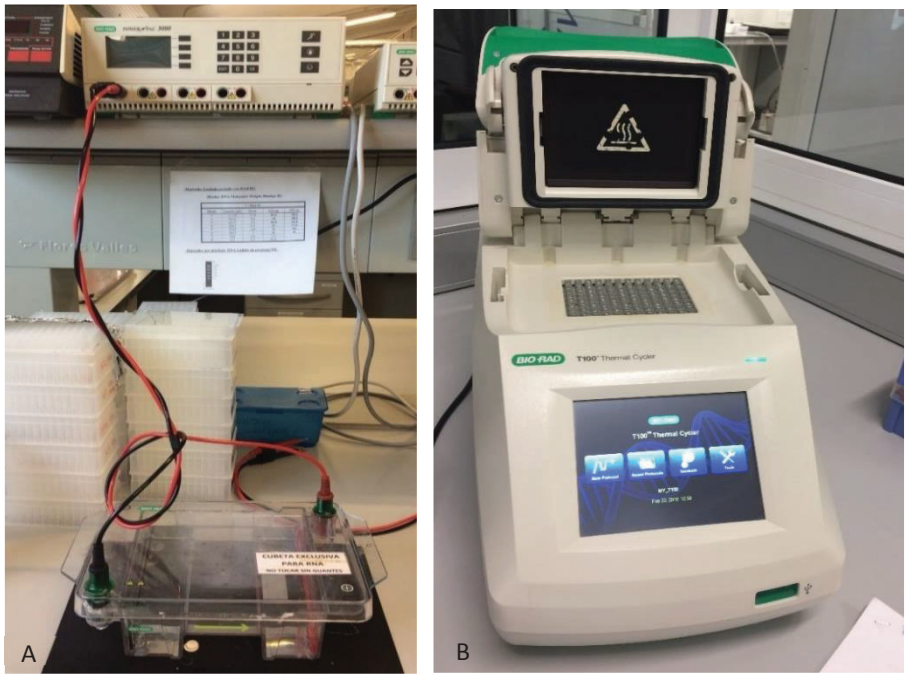

Figure 9. A) Electrophoresis cuvette, B) Thermal cycler (Bio-Rad)

The quantity of the RNA was calculated with a nanodrop® program at an absorbance of 260 nm. If the RNA concentration is higher than 1700 ng/µl, the samples should be diluted.

5.3. CDNA synthesis

Before the complementary DNA (cDNA) synthesis a dilution of the RNA should be made to leave it at 0.5 µg/µl of concentration. Then, this concentration is brought to 10 µl adding the rest with mili Q water, this is the diluted RNA (RNAd). Later, 2 µl of RNAd and 13 µl of water free of RNAses are laid in a plate for subsequent denaturation through a thermal cycler (10 minutes at 65ºC). Next, the cDNA mix is prepared containing 4 µl buffer and 1 µl enzyme transcriptase multiplied by the number of samples, the blank and 2 errors. Once the plate sample has been denatured, in the same plate 5 µl of the cDNA mix are added to the same plate, with a final volume of 20 µl. The plate is put back into the thermal cycler, incubating at 25ºC for 5 min, 42ºC for 30 min and 85.1ºC for 5 min, here the messenger RNA (RNAm) becomes pure cDNA through reverse transcription. Then the standards are prepared with a pool of the cDNA making dilutions 1:5, 1:10, 1:20, 1:50, 1:100, 1:200 and 1:500. Finally, a 1:10 dilution of pure cDNA is made.

5.4. Real Time PCR

The number of transcript copies of *CuZnsod* and *cat* genes was quantified by using (Bio-Rad) real time PCR technique with the standard curve method. Before doing the PCR, first a plate for the constitutive test must be made to find out which is the best primer (*betactine* or *rpl27*) to make the final plate with the gene to analyse. Then a total of 15 µl is placed on the final plate of which 13 µl are the mix prepared with sybergreen, miliQ water and the reverse (R) and forward (F) primers of the gene, and the remaining 2 µl are the DNA samples, the standards previously prepared and the blanks.

Polymerase Chain Reaction (PCR) is divided into three cycles: (i) denaturation, here the DNA chain is separated at 94 °C; then the amplification includes (ii) hybridization, in this process the primers are stuck on the extremes (3' or 5') of DNA chain; and (iii) elongation, here the sybergreen, that contains taq, dntps and a buffer solution of magnesium chloride ($MgCl_2$), synthesizes the inside of the chain. Real time PCR conditions were: 3:30 min at 95ºC, followed by 40 cycles consisting of 15 s at 95°C, 30 s at 58.1ºC and 30 s at 72ºC, then 1 min at 95ºC and 75 cycles of 10 s at 58 ºC. The Ct values obtained by real time PCR amplification were used to create standard curves for target genes. The nucleotide sequence of primers for the genes studied can be seen in Table 1.

Table 1. Nucleotide sequences of primers used for real-time PCR

Gene	**Nucleotide sequence (5'-3')**
bact	FW: TCTGTCTGGATCGGAGGCTC
	RV: AAGCATTTGCGGTGGACG
rpl27	FW: ACAACTCACTGCCCCACCAT
	RV: CTTGCCTTTGCCCAGAACTT
CuZnsod	FW: TTGGAGACCTGGGCAACGTGA
	RV: TCCTCGTTGCCTCCTTTTCCC
cat	FW: ATGGTGTGGGACTTCTGGAG
	RW: AGTGGAACTTGCAGTAGAAAC

6. Statistical analysis

All the data obtained were statistically analysed with Microsoft Excel (Version 1804) and SPSS Statistics (Version 24, IBM Corp., Chicago, IL, USA), where the mean and standard deviation were calculated for all the parameters. In SPSS first data were tested for normality with the one-sample Kolmogorov-Smirnov and Shapiro-Wilk tests. Then, for normally distributed data (if significance>0.05) test of homogeneity of variances and one-way analysis of variance (ANOVA) were used to determine the effects of the different diets, if significance<0.05 means that there are differences between diets. This difference is seen with a post-hoc analysis using Scheffe test. Finally, in SPSS the regressions have been calculated to estimate the relationships between the different variables (dependents) with the diet (independent variable).

In Excel and SPSS quadratic and linear regressions were used to establish a relation between the Cu level in diet and their effect on the different biomarkers. In addition, the Livak method has been used in the statistical analysis for gene expression. This is a relative method, so to do the statistics it has to be compared with a control, which in this case was the diet with the lowest Cu content (6 mg Cu/kg).

RESULTS

1. Feeding trials

All diets were well accepted by the fish and no mortality occurred during the experimental period. All fish grew very well, with an average weight gain of 174.60±6.85 g, SGR 2.40±0.06 %, FCR 1±0.03 g, FE 1±0.03 g and TGC 4.14±0.16 g/days × T (Table 2). No significant differences (p>0.05) were found.

Table 2. Productive parameters data of gilthead seabream fed increasing contents of copper for 42 days

Dietary Cu (mg/kg)	6.00	7.00	9.00	13.00	35.00
Survival (%)	100	98±1	95±5	100	96
SGR (%)	2.43±0.22	2.41±0.10	2.45±0.16	2.30±0.20	2.42±0.07
FCR (g)	1.00±0.09	0.99±0.06	0.97±0.07	1.05±0.06	1.00±0.02
WG (%)	178.5±25.7	175.6±11.1	180.1±19.0	162.8±21.80	176.0±8.40
FE (g)	1.00±0.08	1.01±0.06	1.03±0.08	0.95±0.05	1.00±0.02
TGC (g/days × T)	4.18±0.73	4.18±0.33	4.29±0.34	3.86±0.51	4.19±0.05

2. Proximate and fatty acid composition

At the end of the trial no significant differences were found in the percentage of proteins, lipids and ash contents in seabream fingerlings whole body (Table 3). However, significant differences have been found in moisture content, its highest value is when there is 7 mg Cu/kg (72.99%) and was significantly (p<0.05) higher than those in fish fed with 6 mg Cu/kg (68.92%).

Table 3. Summary of biochemical composition (% fresh weight) in whole body of gilthead seabream fed increasing contents of copper for 42 days

Dietary Cu (mg/kg)	6.00	7.00	9.00	13.00	35.00
% 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
% Moisture	68.92 ± 1.20^{a}	72.99 ± 0.05^{c}	72.19 ± 0.82^{bc}	70.39 ± 0.72^{ab}	70.80 ± 0.91^{b}
% Ash	2.32±0.50	2.66±0.08	2.70±0.22	2.56±0.31	2.43±0.81

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

The most abundant fatty acid was oleic acid (18:1n-9), followed by palmitic acid (16:0), linoleic acid (18:2n-6) and docosahexaenoic acid (22:6n-3). No significant differences ($p<0.05$) were found in the fatty acid composition of whole body from fish fed different Cu levels (Table 4). Increase in dietary Cu up to 9 mg/kg lead to the elevation in polyunsaturated fatty acids from n-6 (r=0.83) and, particularly, n-3 (r=0.62) families, leading to the reduction in oleic acid/n-3 HUFA (r=0.85) and EPA/DHA (r=0.85) as well as an increase in ARA/EPA (r=0.98). However, further elevation of dietary Cu up to 35 mg/kg caused the contrary effect.

Table 4. Fatty acid composition in whole body of gilthead seabream fed increasing contents of copper for 42 days

Dietary Cu (mg/kg)	6.00	7.00	9.00	13.00	35.00
14:0	1.26±0.15	1.35±0.24	1.06±0.23	1.50±0.21	1.22±0.18
14:1n-7	0.08±0.07	0.04±0.01	0.13±0.07	0.04±0.01	0.06±0.00
14:1n-5	0.06±0.04	0.04±0.00	0.13±0.08	0.05±0.01	0.06±0.02
15:0	0.16±0.01	0.16±0.02	0.17±0.04	0.18±0.01	0.16±0.01
15:1n-5	0.01±0.01	0.03±0.01	0.06±0.10	0.01±0.00	0.01±0.01
16:OISO	0.06±0.03	0.05±0.01	0.15±0.11	0.04±0.01	0.07±0.02
16:0	15.37±1.40	15.69±1.09	13.71±1.23	16.13±1.16	15.32±0.83
16:1n-7	3.76±0.30	3.88±0.25	3.39±0.32	3.99±0.32	3.77±0.25
16:1n-5	0.10±0.02	0.14±0.07	0.12±0.04	0.10±0.02	0.10±0.00
16:2n-6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
16:2n-4	0.21±0.07	0.19±0.02	0.20±0.04	0.19±0.04	0.19±0.05
17:0	0.20±0.02	0.19±0.04	0.24±0.01	0.22±0.00	0.21±0.02
16:3n-4	0.22±0.08	0.21±0.03	0.23±0.03	0.19±0.00	0.20±0.04
16:3n-3	0.08±0.02	0.10±0.02	0.13±0.04	0.09±0.02	0.10±0.04
16:3n-1	0.10±0.01	0.10±0.00	0.18±0.07	0.09±0.01	0.13±0.02
16:4n-3	0.18±0.03	0.20±0.02	0.19±0.04	0.19±0.02	0.20±0.07
16:4n-1	0.01±0.01	0.03±0.03	0.00±0.00	0.03±0.03	0.02±0.04
18:0	4.76±0.40	4.72±0.07	4.53±0.31	4.72±0.10	4.72±0.08
18:1n-9	32.57±1.68	32.60±0.16	31.47±1.49	32.69±0.24	32.70±0.68
18:1n-7	2.84±0.09	2.95±0.01	2.92±0.08	2.89±0.09	2.94±0.05
18.1n-5	0.10±0.01	0.09±0.02	0.14±0.08	0.10±0.01	0.10±0.03
18:2n-9	0.74±0.03	0.75±0.04	0.76±0.04	0.74±0.09	0.75±0.10
18:2n-6	11.77±0.34	11.75±0.06	11.39±0.38	12.08±0.04	11.69±0.20

18:2n-4	0.14±0.02	0.12±0.01	0.15±0.16	0.11±0.01	0.15±0.02
18:3n-6	0.46±0.03	0.49±0.02	0.52±0.11	0.45±0.01	0.48±0.05
18:3n-4	0.16±0.03	0.19±0.05	0.24±0.07	0.21±0.08	0.19±0.00
18:3n-3	3.69±0.07	3.59±0.09	3.54±0.09	3.70±0.05	3.60±0.03
18:3n-1	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.01	0.00±0.00
18:4n-3	0.60±0.05	0.61±0.01	0.68±0.07	0.62±0.02	0.58±0.01
18:4n-1	0.09±0.02	0.11±0.02	0.15±0.06	0.10±0.02	0.10±0.02
20:0	0.37±0.02	0.39±0.04	0.45±0.03	0.34±0.03	0.41±0.05
20:1n-9	0.33±0.06	0.34±0.03	0.39±0.11	0.31±0.05	0.32±0.02
20:1n-7	1.80±0.14	1.86±0.02	2.01±0.14	1.81±0.23	1.84±0.11
20:1n-5	0.20±0.04	0.29±0.06	0.30±0.10	0.21±0.07	0.25±0.03
20:2n-9	0.56±0.03	0.58±0.09	0.66±0.02	0.52±0.02	0.54±0.10
20:2n-6	0.33±0.03	0.36±0.02	0.41±0.06	0.36±0.08	0.35±0.05
20:3n-9	0.03±0.02	0.04±0.01	0.08±0.08	0.05±0.00	0.10±0.01
20:3n-6	0.23±0.12	0.22±0.03	0.38±0.05	0.24±0.04	0.27±0.01
20:4n-6	0.57±0.06	0.56±0.03	0.68±0.08	0.55±0.02	0.60±0.09
20:3n-3	0.19±0.05	0.16±0.05	0.23±0.04	0.20±0.09	0.20±0.02
20:4n-3	0.46±0.06	0.49±0.06	0.53±0.07	0.44±0.04	0.46±0.06
20:5n-3	3.60±0.45	3.50±0.34	3.88±0.33	3.44±0.11	3.51±0.24
22:1n-11	1.35±0.26	1.35±0.05	1.65±0.18	1.24±0.20	1.44±0.21
22:1n-9	0.62±0.15	0.64±0.09	0.76±0.51	0.51±0.06	0.69±0.12
22:4n-6	0.27±0.06	0.26±0.03	0.30±0.06	0.29±0.07	0.30±0.00
22:5n-6	0.31±0.08	0.28±0.04	0.30±0.05	0.25±0.05	0.30±0.03
22:5n-3	1.85±0.54	1.75±0.18	2.14±0.28	1.62±0.20	1.77±0.35
22:6n-3	7.17±1.99	6.55±0.74	8.27±1.04	6.16±0.63	6.84±1.09
Saturated	22.16±1.92	22.56±1.22	20.30±1.57	23.13±1.34	22.12±1.05
Monoenoics	43.83±1.50	44.25±0.15	43.47±0.66	43.97±0.49	44.29±0.52
n-3	17.82±3.15	16.95±1.29	19.59±1.77	16.44±1.04	17.25±1.61
n-6	13.95±0.16	13.92±0.10	13.98±0.29	14.22±0.22	13.98±0.07
n-9	34.73±1.40	34.73±0.30	34.12±1.33	34.82±0.13	35.10±0.65
n-3HUFA	5.15±0.23	5.10±0.12	5.54±0.29	5.22±0.24	5.26±0.06
AA/EPA	0.16±0.01	0.16±0.01	0.17±0.01	0.16±0.00	0.17±0.01
EPA/DHA	0.52±0.07	0.54±0.01	0.47±0.02	0.56±0.04	0.52±0.05
oleic/DHA	4.80±1.36	5.02±0.51	3.85±0.62	5.35±0.59	4.87±0.82
oleic/n-3HUFA	6.34±0.60	6.39±0.14	5.70±0.57	6.27±0.32	6.22±0.06
n-3/n-6	1.28±0.22	1.22±0.09	1.40±0.11	1.16±0.06	1.23±0.12

3. Histology

Histology study of liver seabream fingerlings denoted that increase in dietary Cu contents up to 35 mg/kg significantly (p<0.05) increased hepatic steatosis, up to 13 mg/kg elevated the degree of sinusoids dilatation and up to 9 mg/kg raised both nucleus displacement and occurrence of cells with broken margins. The variables have been evaluated on a 0-3 scale (Fig. 10-12).

Table 5. Summary of histology data of liver gilthead seabream fed increasing contents of copper for 42 days

Dietary Cu (mg/kg)	Steatosis	Peripheral nucleus	Broken cell margin	Sinusoids dilatation
6.00	1.56 ± 0.12^{a}	0.32 ± 0.06^{a}	0.50 ± 0.10^{a}	0.35 ± 0.16^{a}
7.00	1.93 ± 0.07^{ab}	0.72 ± 0.10^{a}	0.64 ± 0.04^{a}	0.38 ± 0.03^{a}
9.00	1.95 ± 0.14^{ab}	1.02 ± 0.03^{b}	0.99 ± 0.13^{b}	0.56 ± 0.06^{a}
13.00	2.11 ± 0.10^{ab}	1.40 ± 0.05^{b}	1.03 ± 0.10^{c}	0.89 ± 0.02^{b}
35.00	2.78 ± 0.07^{b}	1.37 ± 0.18^{b}	1.30 ± 0.03^{c}	1.00 ± 0.06^{b}

Different letters in the same row indicate significant differences, p<0.05, n=5

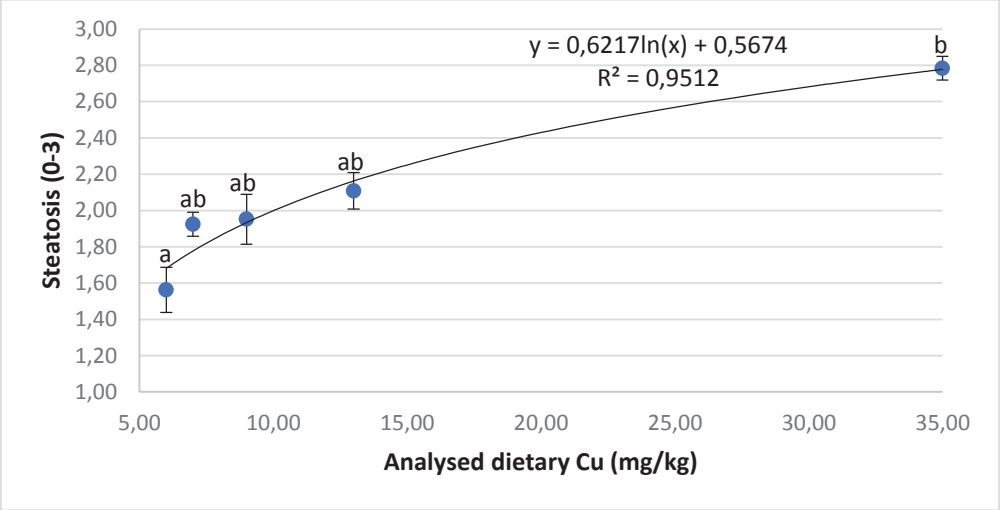

Figure 10. Relation between diet Cu concentration and steatosis level in the liver of gilthead seabream fed increasing contents of copper for 42 days

Different letters indicate significant differences, p<0.05, n=5

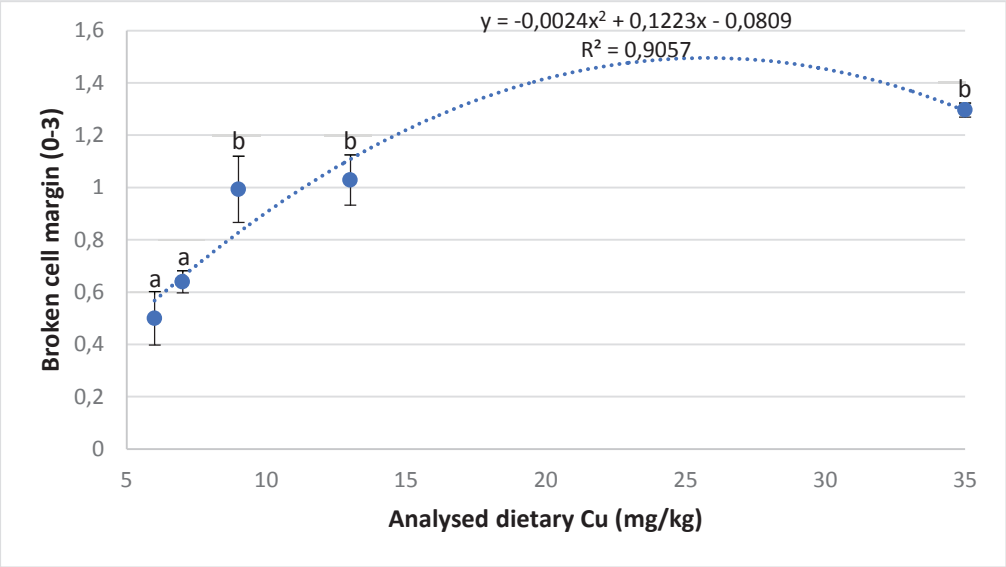

Figure 11. Relation between diet Cu concentration and broken cell margin in liver of gilthead seabream fed increasing contents of copper for 42 days

Different letters indicate significant differences, p<0.05, n=5

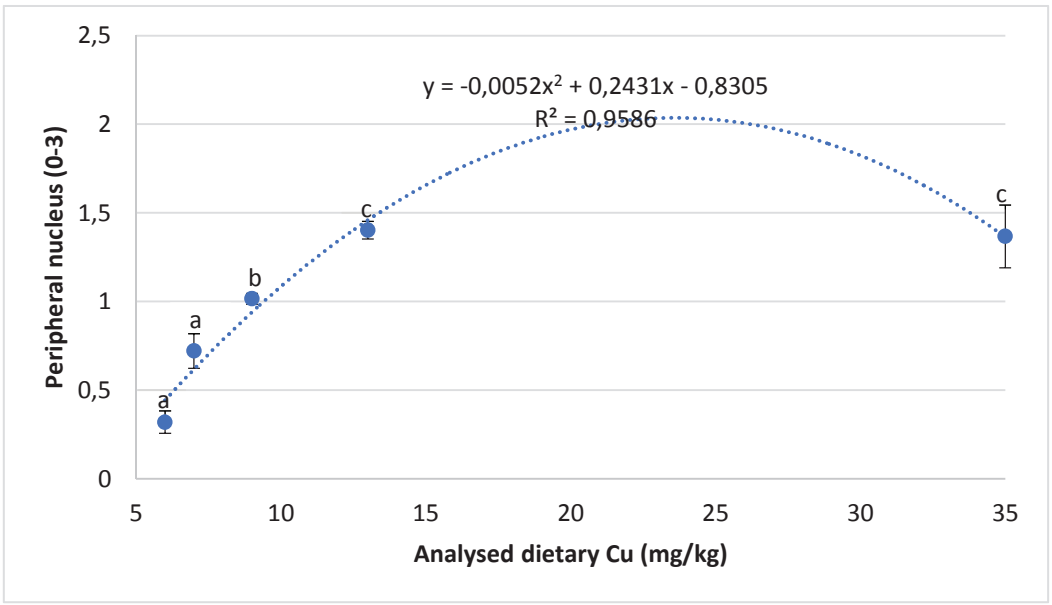

Figure 12. Relation between diet Cu concentration and nucleus location in liver of gilthead seabream fed increasing contents of copper for 42 days

Different letters indicate significant differences, p<0.05, n=5.

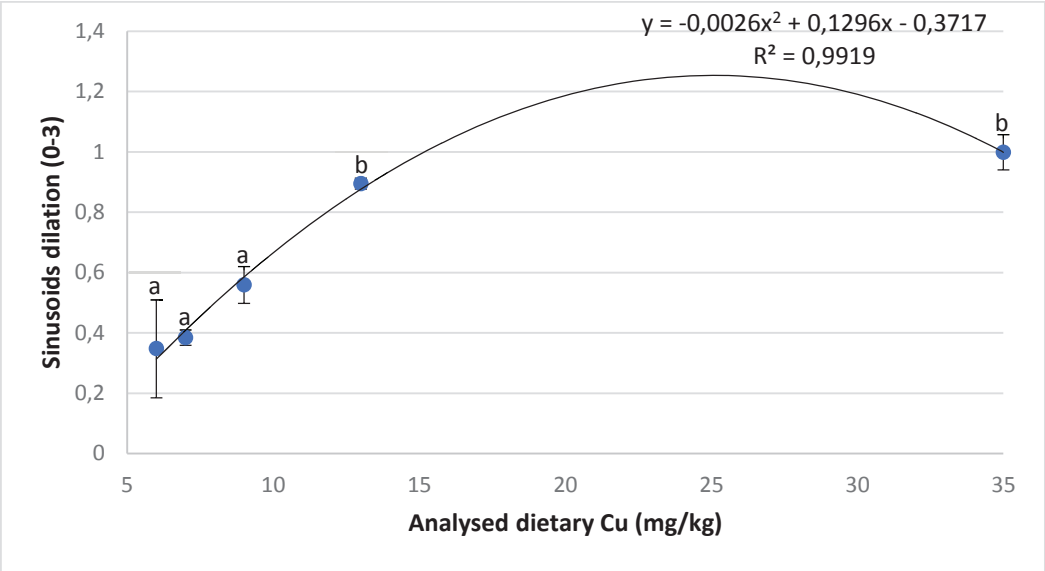

Figure 13. Relation between diet Cu concentration and the sinusoids dilatation in liver of gilthead seabream fed increasing contents of copper for 42 days

Different letters indicate significant differences, p<0.05, n=5.

3. Gene expression

The gene expression data of *CuZnsod* was not significantly (p>0.05) affected by dietary Cu levels (Table 6). However, elevation of dietary Cu up to 9 mg/kg significantly (p<0.05) up-regulated *cat* expression, whereas further elevation caused the opposite effect (Fig. 14).

Table 6. Summary of gene expression data of liver gilthead seabream fed increasing contents of copper for 42 days

Dietary Cu (mg/kg)	*CuZnsod*	*cat*
6.00	1.01±0.18	1.40 ± 1.27^{ab}
7.00	1.20±0.18	3.31 ± 0.55^{b}
9.00	1.06±0.18	4.88 ± 0.19^{b}
13.00	1.12±0.01	1.08 ± 0.38^{a}
35.00	1.21±0.27	1.09 ± 0.68^{a}

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

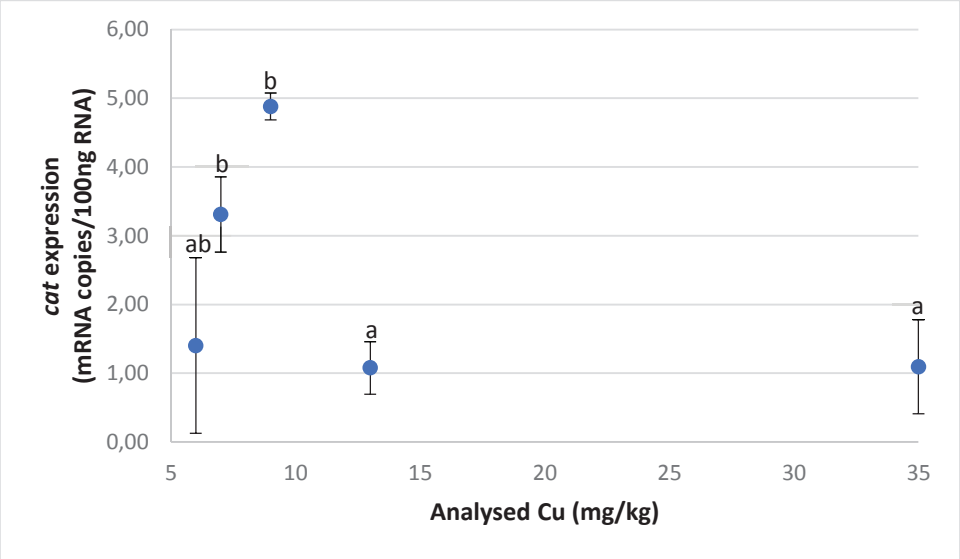

Figure 14. Relation between diet Cu concentration and cat expression in liver of gilthead seabream fed increasing contents of copper for 42 days.

Different letters indicate significant differences, $p<0.05$, $n=3$.

DISCUSSION

A Cu deficiency can produce different negative effects on fish's organism such as a reduction of growth, formation of eye cataracts and altered activities of cytochrome c oxidase in heart and CuZnSOD in liver (Watanabe *et al.*, 1997; Tacon, 1992; Wilson & Gatlin, 1986; Halver & Hardy, 2002). Besides, all body tissues should be negatively affected, since CuZnSOD acts as an antioxidant intercepting and inactivating oxygen free radicals in cells (Betancor, 2012; Shao *et al.*, 2010; Tang *et al.*, 2013). In the present study, the lowest dietary Cu levels tested (6 mg Cu/kg) did not caused low growth or other signs of Cu deficiency. Optimal Cu levels in diet for several fish species ranges from 1.5 to 6 mg Cu/kg diet (NRC, 2011), despite Cu requirements may vary greatly even within the same species depending on life stages (Clearwater *et al.*, 2002). Accordingly, the lowest dietary Cu levels tested in this study were not enough to observe a Cu deficiency and would be enough to meet the needs of seabream fingerlings. Nevertheless, dietary increase from 6 to 9 mg Cu/kg, lead to a generalized increase in n-3 HUFA and slight improvements in productive parameters such as WG, FE, SGR and TGC, as well as an up-regulation of *cat* gene expression. Moreover, no negative effects were observed in hepatocytes morphology. These results are in agreement with the higher WG and FE obtained in other fish species when fish were fed intermediate Cu levels in comparison to those fed ≤1.66 or ≥11.03 mg Cu/kg diet (Lin *et al.*, 2008). Similarly, highest CAT activity has been also found in blunt snout bream (*Megalobrama amblycephala*) when fish were fed diets supplemented with 25 mg Cu/kg (Shao *et al.*, 2012) or for young grass carp (*Ctenopharyngodon idella*) when fish were fed diets supplemented with 3.75 mg Cu/kg (Tang *et al.*, 2013). On the contrary, in European sea bass (*Dicentrarchus labrax*) no relationship was found between Cu and CAT activity in kidney (Roméo *et al.*, 2000). However, this up-regulation of *cat* expression in fish fed 9 mg Cu/kg, was not correlated with a higher *CuZnsod* expression.

Excess of Cu may be also harmful for fish and cause structural damages in gill, liver or kidney, as well as behaviour alterations (Carol Ann Woody and Louise, 2012) and growth reduction or reduced feed efficiency (Tang *et al.*, 2013; Watanabe *et al.*, 1997). Moreover, as an accumulative contaminant heavy metal, at high concentrations Cu can be very toxic and even lethal (Tacon, 1992). In this study, increase in dietary Cu levels over 9 mg/kg Cu led to reduction in HUFA levels, down-regulation of *cat* expression and, most importantly, an increase in liver damage, suggesting symptoms of a Cu excess in seabream fingerlings. These symptoms suggested that from 9 mg Cu/kg tissues are less protected from free radicals by CAT activity, denoting that the reduction of peroxide (H_2O_2) to water (H_2O) is not taking place correctly. Indeed, Cu excess is related to the production of oxygen-free radicals, which also could cause widespread damage to hepatocytes such as lipoperoxidation of polyunsaturated membrane lipids with leakage of proteins (Isani *et al.*, 2011). This agrees with the hepatic alterations and damage observed at high Cu concentrations (NRC *et al.*, 2011; Watanabe *et al.*, 1997). The hepatocyte damage could be seen in histological results through an increase of steatosis, peripheral nucleus, break of the cellular margin and the dilatation of sinusoids.

Nevertheless it must be pointed out that, on one hand, steatosis is the fat accumulation in the liver, which can cause the displacement of the cell nucleus and breakage of cell margins in more severe cases. This could be because Cu could enhance the metabolism of lipid synthesis and this induce the increase of hepatic lipid deposition (Chen *et al.*, 2013). On the other hand, Cu tends to accumulate in the liver, eyes, heart and brain (Watanabe *et al.*, 1997; Halver and Hardy, 2002) and its excessive increase may unbalance metals in the organism. 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. Therefore, the liver damage found in gilthead seabream in the present study would confirm an excess of dietary Cu (35 mg/kg). Similarly, rainbow trout exposed to toxic Cu levels also presents increased dilatation of sinusoidal spaces (Handy *et al.*, 1999). Also 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, the decrease in CAT activity from 9 mg Cu/kg may be related to the decrease (not significant) of lipid content when the amount of copper is higher, since it could be the result of an increase in lipid peroxidation of the membranes (Roméo *et al*., 2000; Isani *et al.*, 2011). Similarly to the present study, were was no mortality or significant changes in growth were found in fish fed the highest Cu levels, dietary Cu concentrations up to 350–684 mg Cu/kg diet (Julshamn et al., 1988; Miller et al., 1993; Mount et al., 1994). However, in channel catfish fed 40 mg Cu/kg diet (Wilson and Gatlin, 1986) or Atlantic salmon fed 98 mg Cu/kg diet (Lorentzen *et al.,* 1998) reduced growth or survival.

CONCLUSIONS

- For gilthead seabream fingerlings fed diets with low fishmeal content, Cu basal levels (6 mg Cu/ kg) would be sufficient to cover the requirements for growth and survival.
- To optimize whole body n-3 HUFA contents and oxidative status dietary Cu should be increased at a maximum of 9 mg Cu/kg.
- Dietary Cu levels should never be increased over 9 mg Cu/kg, to avoid the decrease in n-3 HUFA content in whole body and the increase in liver steatosis, broken cell margin, peripheral nuclei and sinusoid dilatation, markers of hepatic damage and cholestasis that denote a potential toxic effect of Cu.

Despite the advances made, it is necessary to continue doing more studies on the requirements of microminerals, especially Cu, since there is currently little documentation about it. As this study has not solved all the doubts generated at the beginning, research is still on-going on the effect that dietary Cu could have on the TBARS of seabream fingerlings, to know if oxidation is taking place. Other line of work to be developed in the future and make this type of research more sustainable and respectful, is to be able to evaluate the concentrations of copper in non-invasive tissues, since, for example, currently blood samples do not give a clear or measurable result with which to work.

ANNEXES

1. Abbreviations

ARA: Arachidonic acid

CuZnSOD: Copper zinc superoxide dismutase

CAT: Catalase

CDNA: Complementary desoxiribonucleic acid

DHA: Docosahexaenoic acid

DNA: Desoxiribonucleic acid

EPA: Eicosapentaenoic acid

FAMES: Fatty Acid Methyl Esters

FM: Fish meal

FO: Fish oil

Seabream: Gilthead seabream

HUFA: High Unsaturated Fatty Acids

PCR: Polymerase Chain Reaction

RNA: Ribonucleic acid

TBARS: Thiobarbituric acid reactive substances

VM: Vegetable meal

VO: Vegetable oil

WB: Whole Body

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• Descripción detallada de las actividades desarrolladas durante la realización del TFT

En primer lugar, antes de poder ponerme a trabajar con mis muestras dentro del laboratorio, realicé otro tipo de tareas de acondicionamiento como realizar muestreos en los que medía y pesaba peces (normalmente dorada), buscar bibliografía relacionada con el trabajo y estudio que iba a realizar, y, finalmente, aprender lo que debería hacer posteriormente en el laboratorio.

Una vez aprendido esto, procedí a trabajar con mis muestras. Empecé en el laboratorio de bioquímica, en el cual analizaba la composición proximal (cuantificación de lípidos, proteínas, humedad y cenizas crudas) y los ácidos grasos (mediante metilación) de muestras de pez entero homogenizado de juveniles de dorada*.

En el laboratorio de genética, con las muestras de hígado de los juveniles de dorada realicé una extracción de ARN (con un pool de tres hígados por tanque). Seguidamente realicé un chequeo del ARN extraído en el que cuantificaba la cantidad de ARN de la muestra mediante el nanodrop y visualizaba la calidad del ARN mediante electroforesis*. Después hice la síntesis de cDNA, mediante la cual el ARN que había extraído se transforma en cDNA después de varios procesos*. Una vez tenía sintetizado el cDNA en una placa lo pasaba a otra placa, en la cual cada pocillo debía tener un total de 15 µl: 13 µl de un mix (de sybergreen, agua miliQ y los primers del gen a analizar) y 2 µl de la muestra de cDNA, estándares o blancos. Una vez preparada la placa procedía a obtener la expresión del gen a evaluar mediante una PCR a tiempo real*, primero hice el gen *CuZn-superóxido dismutasa* y después la *catalasa*.

Finalmente, en el laboratorio de histología procedí a evaluar la presencia de esteatosis, rotura del margen celular, desplazamiento del núcleo de la célula y la dilatación de los sinusoides del hígado de juveniles de dorada. Para ello primero extraje 5 hígados de distintos peces por tanque, los segmenté y los puse en cassettes histológicos. Después estos casetes pasaron por un procesador* para deshidratar y embeber las muestras en parafina. Seguidamente pasé las muestras a un bloque con parafina, el cual tallé y corté para obtener secciones delgadas en las

que se pudieran observar las muestras. Estas secciones se adhieren en un portaobjetos que posteriormente teñí con una tinción de hematoxilina y eosina. Una vez preparados los portaobjetos procedí a evaluar al microscopio las distintas variables en una escala 0-3.

Una vez realizadas todas las tareas necesarias para obtener los resultados del trabajo, me enfoqué mediante el análisis estadístico y leyendo artículos científicos similares para razonar los resultados obtenidos y así realizar el documento del TFG.

**Para una descripción más detallada, puede consultar el apartado de material y métodos del trabajo.*

• Formación recibida (cursos, programas informáticos, etc.)

La formación recibida durante el trabajo de fin de grado (TFG) realizado ha sido bastante variada. Principalmente, mi trabajo se ha centrado en la parte de laboratorio y cada vez que entraba a un departamento distinto antes de ponerme a realizar las tareas en solitario las jefas técnico de laboratorio me explicaban y acompañaban durante los primeros días. Estando en el laboratorio he aprendido muchas cosas y utilizado máquinas que nunca había hecho antes, especialmente en los laboratorios de genética e histología. Además he aprendido a utilizar de manera más desenvuelta el programa SPSS a la hora de analizar estadísticamente los datos de los resultados obtenidos.

• Nivel de integración e implicación dentro del departamento y relaciones con el personal.

Con respecto a la relación con el personal podría decir que ha sido muy buena, tratándonos entre nosotros de una forma cordial. Mi nivel de implicación dentro del departamento creo que ha sido bastante alto, ya que siempre me han tratado como a uno más y he realizado las mismas tareas que el resto del personal.

• Aspectos positivos y negativos más significativos relacionados con el desarrollo del TFT

En cuanto a los aspectos positivos cabe destacar su filosofía de trabajo, el buen nivel de integración en la empresa, las relaciones con el personal, el aprendizaje que he recibido y el poder colaborar en un proyecto tan importante como PerformFISH, además de poder asistir a un congreso internacional de acuicultura (ISFNF).

Sin embargo, por poner un aspecto negativo, he tenido que aplazar la entrega del TFG (Junio a Julio), ya que el volumen de trabajo era mucho y además surgieron problemas típicos del día a día, como máquinas estropeadas, reactivos contaminados, etc, por lo que no daba tiempo de redactarlo en condiciones para esa primera fecha.

• Valoración personal del aprendizaje conseguido a lo largo del TFT.

Durante la realización del TFG me he dado cuenta de la multidisciplinariedad de la carrera dado que he podido ver y poner en función varias de las enseñanzas recibidas durante la carrera, como pueden ser muchas de las aprendidas durante asignaturas como sobre todo la de acuicultura y biotecnología marina, además de otras muchas asignaturas relacionadas como recursos vivos marinos, pesquerías, química marina, fisiología de los organismos marinos, estadística, etc.

Aunque, por otra parte, también me he dado cuenta de que he aprendido muchas cosas que no sabía antes como puede ser especialmente de patología animal, genética, etc. Además, he podido ver cómo es investigar y publicar un artículo científico, gracias a que el trabajo realizado está enmarcado dentro de un proyecto de investigación europeo como es PerformFISH. En conclusión, estoy bastante satisfecha con el trabajo realizado durante mi TFG, ya que me he sentido útil en la sociedad, aportando un granito de arena en este tipo de investigaciones, las cuales intentan que la acuicultura sea aún más sostenible.