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A MICROSCOPE AND IMAGE ANALYSIS STUDY OF THE LIVER AND EXOCRINE PANCREAS OF SEA BREAM *Sparus aurata* FED DIFFERENT DIETS

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Abstract- The liver is a target organ for applying nutritional research studies. The aim of the present study is to determine the effects of different fish diets in Sparus aurata's liver morphology. S. aurata were fed the experimental diets for 41 days. Tissue liver samples were obtained from three fish of each dietary treatment, fixed, dehydrated and embedded in resin blocks. Semi-thin sections (2-3 μm) stained with toluidine blue. Ultra-thin sections (40-80 nm) were prepared for T.E.M. observation. The general morphology of all the examined samples was normal. All the samples had exocrine pancreatic islets with similar number of secretory zymogen granules in acinar cells. The number similarity of the secretory zymogen granules suggests that all the examined fishes irrespectively of their dietary treatment were in similar nutritional condition. However, this study shows fish fed a commercial diet had larger lipid droplets in their liver compared with those fed the other two diets. The commercial diet contained higher percentage of lipids, higher percentage of fish oil and plant ingredients that are not digestible and contain anti - nutritional factors (substances - protease inhibitors). In sea bream, steatosis can result either by an increase in the dietary lipid content or an essential fatty acid deficiency or by the use of vegetable oils in the diets.

Keywords- *Sparus aurata*, liver, lipid droplets, exocrine pancreas, microscopy, image analysis

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Introduction

Gilthead sea bream (*Sparus aurata*) is a carnivorous marine fish which belongs to the sparidae family. Sparidae aquaculture accounted for 244.153 tonnes of fish in 2006 [1]. Greece is the leading producer in the Europe [2].

The liver may be a localized organ in the anterior abdomen or may, in some species, have processes which extend the length of the abdomen or are closely applied to the other viscera. In teleost fish (*S. aurata* included) the exocrine pancreatic tissue is developed around the hepatic portal vein. In *Sparus aurata* the exocrine pancreas or hepatopancreas has an acinar arrangement and separated by the hepatic parenchyma by a thin layer of connective tissue [3,4]. Ultrastructually, the hepatocyes of *S. aurata* are polygonal and have a spherical nucleus with euchromatin and heterochromatin, prominent nucleolus, nuclear membranes and pores [4]. In fishes there is much less tendency of the hepatocytes to form distinct cords in the hepatopangreatic parenchyma. The formed cords have a one or two cells thickness [5]. The liver of *S. aurata* has large contents of lipids and a well-developed biliary tract. Biliary tract structures were associated with dietary habits. In cultured fish, hepatocytes are often swollen with glycogen or neutral fat. Triacylglycerol and cholesteryl esters are stored in lipid droplets. Up to 70% of the triacylglycerol substrate for the assembly of very low density lipoproteins is provided by the hepatic lipid droplet pools. The remainder is coming from de novo synthesis [6]. In mammals, lipid droplets within retinoid stellate cells in the liver contain the majority of the body's vitamin A and its metabolites [7]. The number and size of these lipid droplets is influenced chiefly by the amount of dietary retinoid intake [8].

The pancreatic tissue is more variable in location, even within a single species. It can be found as islets of secretory tissue among the fat cells in the mesentery of the pyloric caeca, as part of the spleen and as islets around the hepatic portal vein [9]. In salmonids, it is diffused throughout the tissue (adipose) that surrounds the pyloric caeca. In catfish, bass and sea bream, it surrounds the portal vessels entering the liver to form a hepatopancreas [9]. The structure of the pancreatic tissue is very similar to that of the mammal and consists of endocrine (islets of Longerhans) and exocrine parts. The exocrine part is comprised of acinar cells that have a large spherical nucleus with 1-3 nucleoli [5,9].

Liver is the main organ in which the lipid metabolism, synthesis and degradation of fatty acids are regulated [10,11]. One of the main functions of *S. aurata* liver is that it is the main energy reservoir [12]. The use of the liver as an indicator organ of the nutritional and physiological status in fish is well-known [13,14]. The capacity of the hepatic cells to oxidize fatty acids is limited. When there is excessive dietary intake of lipids, large synthesis and deposition of triacylglycerols in vacuoles take place. This morphological liver

pattern is known as steatosis. In cultured sea breams steatosis may be the result of increment of lipid content in the dietary due to the use of artificial diets [15,16]. Excessive lipid accumulation or steatosis of hepatocytes is generally regarded as a pre-neoplastic toxicopathic change in mammalian studies, but its significance in fish is less certain.

The present study aims to examine the effect of different dietary regimes with different lipid contents and different proportion of plant and fishmeal proteins to the *S. aurata* livers through photon microscopy, electron microscopy and image analysis.

Materials and Methods

Sea breams (*S. aurata*) were obtained from a commercial fish farm in Greece. A detailed description of the growth experimental setup can be found in Berillis, et al [17] and Carter, et al [18]. In brief, sea breams, initial average weight of 35.24 ± 1.47 g, were distributed in groups of 25 fish in nine tanks of 120 l, in triplicate per treatment and left to acclimate for two weeks. Fish were fed the experimental diets ad libitum by hand three times per day (09:00, 12:00, and 15:00), 7 days/week for 41 days. The experimental diets were isoenergetic. Diet A, an organically produced feed including sustainable certified fish meal (63%), fish oil (9%), and organic wheat (26.5%); Diet B, a laboratory diet including fish meal (55%), fish oil (9%), wheat meal (25.2%) and soya meal (10%) and Diet C, a commercial diet with fish meal (50%), fish oil (14%), wheat meal (11.7%), soya meal (16%) and corn gluten meal (7.5%) were used. The lipid content of diet A, B was 14%, while for diet C was 17%. At the end of the experiment, fish were weighed and sacrificed by immersion in ice cold water (hypothermia). Their body cavity was opened through a midventral incision and tissue liver samples were immediately obtained from three fish of each dietary treatment. The liver samples were divided equally; the middle part was taken for measurement, and immediately fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate solution until further analysis.

For electron microscopy and semi-thin section microscopic examination, tissue specimens were prefixed in 2.5% glutaraldehyde diluted in a 0.1M sodium cacodylate solution for 24 hrs. at 4°C. The specimens were washed in the same buffer before and after fixation. Post-fixation was performed with 1% osmium tetroxide in 0.1 M sodium cacodylate for 2 hrs. at 4°C. The specimens were washed in the same buffer before and after post-fixation and then rinsed in distilled water. Then, the specimens were dehydrated in a graded ethanol series and were subsequently immersed and left overnight in a 1:1 mixture of propylene oxide and the embedding resin. The final step of the embedding took place in capsules containing agar resin. Polymerisation of the resin was completed after 48 hrs. at 60° C. Semi-thin sections were cut with a Reichert Supernova ultramicrotome (DeKalb, IL, USA). These sections (2-3 μm) were stained with toluidine blue and examined under a Radical RMH-4B pathological microscope. Images were taken with an attached to the microscope digital camera. Ultrathin sections (60 to 80 nm) were cut with the same Reichert Supernova ultramicrotome, mounted on copper grids, stained with uranyl acetate and lead citrate and examined in a Philips CM10 electron microscope (Amsterdam, The Netherlands).

A special image analysis algorithm was created in Matlab® (MathWorks®, U.S.A.), in order to calculate automatically the area of the liver's lipid droplets. Minimum 5 images from each group (RGB mode, 300 dpi) acquired with photon microscope's digital camera were used. Lipid droplets were represented as white objects against a black background. A vertical and horizontal scanning of the images was performed in order to measure the area of each object. This area was measured in pixels, which were subsequently converted to micrometers using the dpi of the transferred image. At least 200 lipid droplets per diet were measured. 10 acinar cells of pancreatic islets per diet were randomly chosen and the number of the secretory zymogen granules was measured.

Lipid droplets and secretory zymogen granules area values were checked for normality using the Kolmogorov-Smirnov and Shapiro-Wilk test respectively. Statistical comparisons between the groups were made using the Mood's Median Test for the non-normal distributed data and with one-way ANOVA for the normal distributed data.

Results

Photomicroscopy examination of liver semi thin sections does not reveal any histological abnormality for all of the three diets that the sea breams were fed [Fig-1], [Fig-2], [Fig-3]. The majority of the nuclei appeared in the center of the hepatocytes, having prominent heterochromatin and nucleolus. The observed distinct cords had the normal thickness (one or two cells). No hemorrhagic symptoms were observed. The pancreatic islets contained acinar cells with many secretory zymogen granules. The number of these granules was similar for all of the used diets [Table-1]. Lipid droplets area size data found not to be drawn from a normal distribution (p<0.05). Diets A and B seem to form less and significant smaller lipid droplets in the hepatic parenchyma than Diet C [Table-1]. The area range was also bigger when the conventional diet was used [Table-1]. Steatosis was not detected in the liver tissues of the three dietary regimes.

Ultrastructure examination with transmission electron microscope revealed the intact nuclear envelope and the euchromatin of the nuclei. The secretory zymogen granules of the acinar cells were visible in a more clear and efficient way. They appeared electron dense, revealing that they contained digestive enzymes in order to break down proteins, fats, and carbohydrates [Fig-4].

Table 1- Mean size of S. aurata liver lipid droplets and number of secretory zymogen granules per acinar cell fed the experimental diets

Diets	Lipid droplets area size (μm²)	Lipid droplets area size range max-min (μm²)	Number of secretory zymogen granules per acinar cell
C	0.03^{a}±0.03 (n=571)	0.27 (max=0.28, min=0.01)	12.2^{a}±1.8 (n=10)
B	0.03^{b}±0.03 (n=229)	0.14 (max=0.15, min=0.01)	12.4^{a}±0.9 (n=10)
A	0.02^{b}±0.01 (n=242)	0.12 (max=0.13, min=0.01)	16.4^{a}±2.0 (n=10)

Data for lipid droplets area size are presented as median ± interquartile range.

Data for number of secretory zymogen granules per acinar cell are presented as mean ± S.E

Means in a column followed by the same superscript are not significantly different (p > 0.05).

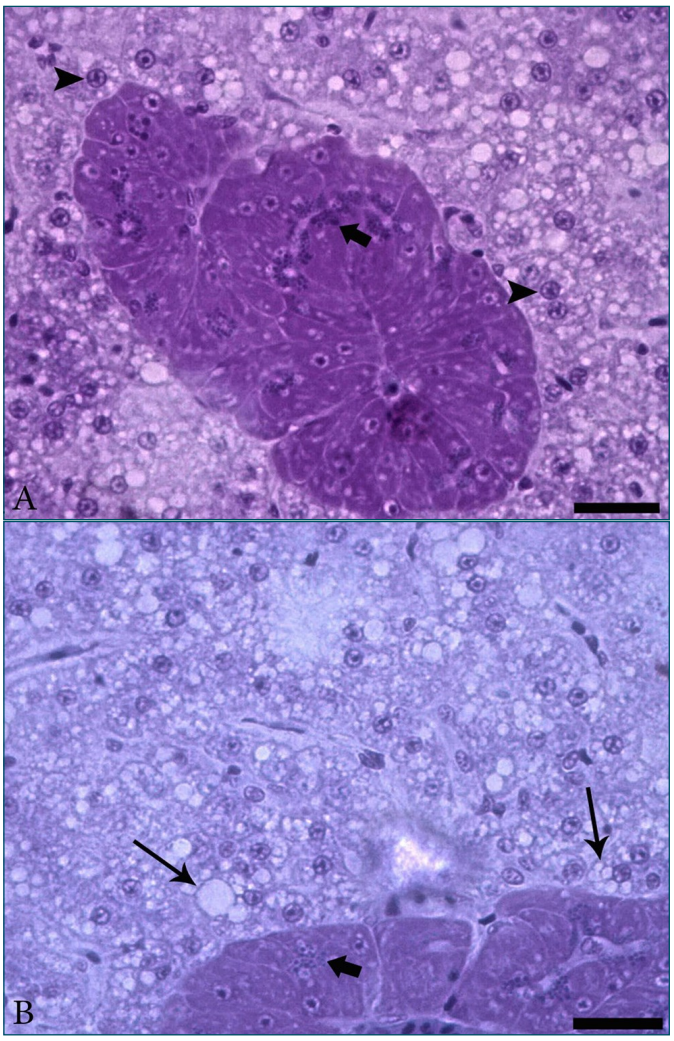

Fig. 1- Histological sections (toluedin blue stain) of *S. aurata* liver, fed with conventional diet C. Big amounts of lipid droplets (arrows) are present. Variant sizes of lipid droplets can also be observed. Nuclei (arrowheads) have normal shape (spherical) and prominent heterochromatin and nucleolus can be observed. Many secretory zymogen granules (small arrows) are present in the exocrine pancreatic islets. Scale bar= 25 μm

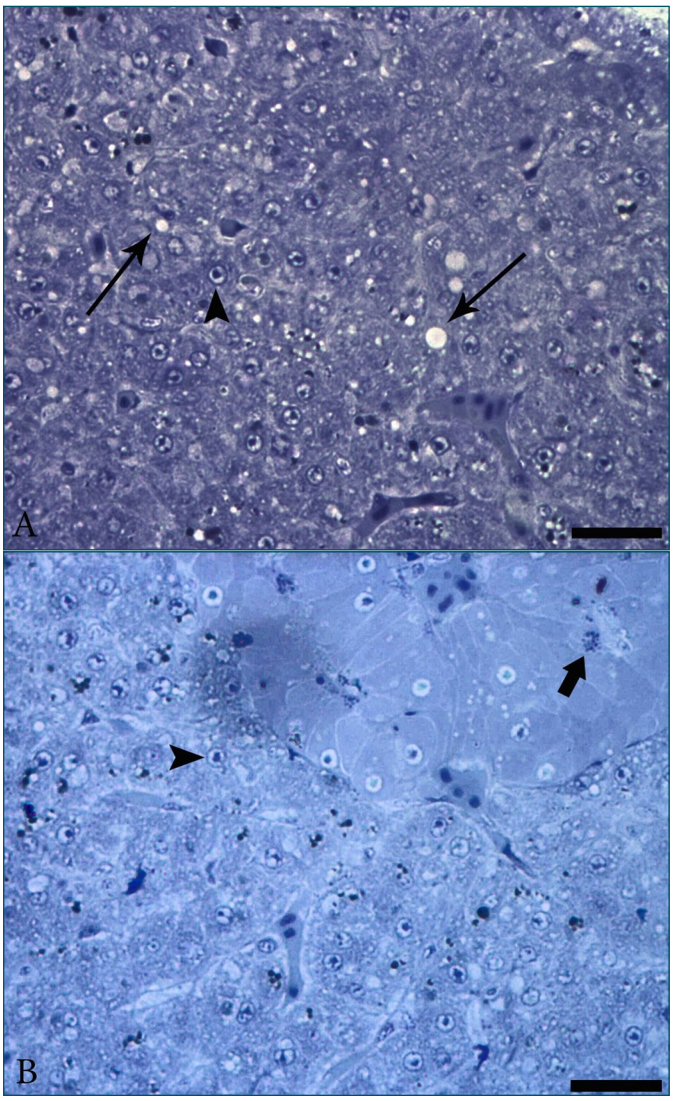

Fig. 2- Histological sections (toluedin blue stain) of *S. aurata* liver, fed with laboratory diet B. Lipid droplets are less in number and in size than fishes fed with commercial. Secretory zymogen granules are also present. Nuclei appeared normal. Scale bar= 25 μm.

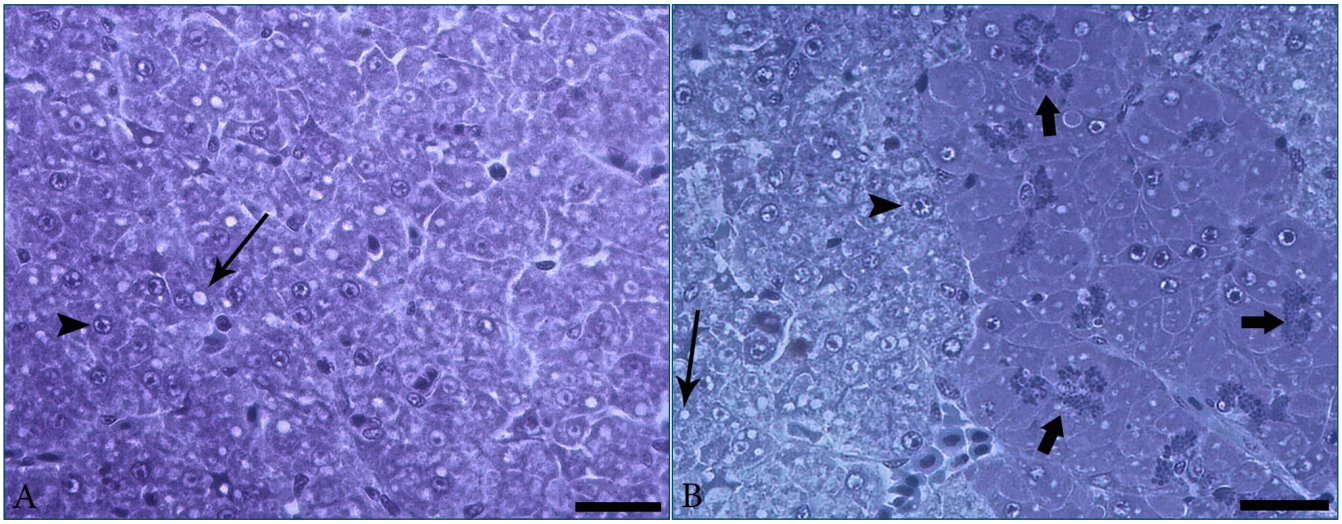

Fig. 3- Histological sections (toluedin blue stain) of *S. aurata* liver, fed with organic diet A. The observations are similar to the laboratory diet fed fishes. Scale bar= 25 μm.

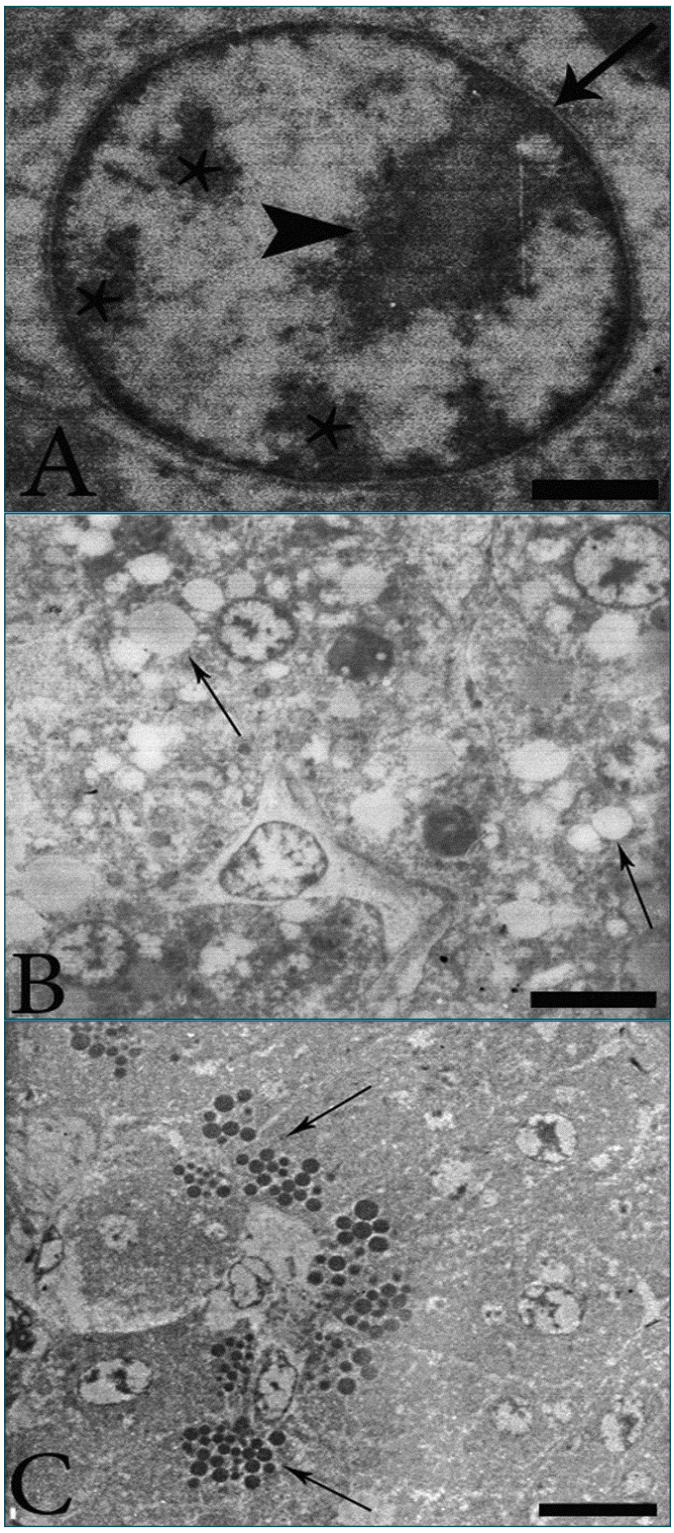

Fig. 4- Electron microscope images from the hepatic parenchyma of *S. aurata*. **(A)** Hepatocyte nucleus from *S. aurata* fed with organic diet A. The nucleus appeared normal with distinct euchromatin within the nucleoplasm, heterocromatin (stars) and nucleolus (arrowhead). Nuclear envelop (arrows) appeared to be intact. Scale bar=1 µm. **(B)** Hepatic parenchyma of *S. aurata* fed with conventional diet C. Many lipid droplets (arrows) can be observed having various sizes. Scale bar=6 µm. **(C)** Pancreatic islet of *S. aurata* fed with laboratory diet B. Arrows are showing the secretory zymogen granules containing digestive enzymes. Scale bar=10 µm.

Discussion

Fish meal and fish oil have been used in the aquafeeds of sea bream aquaculture. However, for sustain-ability and economic reasons it is necessary to rely less on them. Tacon and Metian [19] projected that by 2020 there will be a 44.5% and 15.5% reduction in the use of fish meal and fish oil respectively in aquafeeds. The isonitrogenous diets offered in sea breams (*S. aurata*) were formulated to replace a percentage of fishmeal with alternative plant protein sources; diets contained 63, 55 and 50 % fishmeal; in the latter two diets, part of the fishmeal protein was replaced with plant protein [17,18]. Our results show that when the inclusion of fish oil in the diet is reduced from 14% (commercial diet) to 9% (organic and laboratory diet) and in addition, fish meal inclusion is increased accordingly to produce isoenergetic diets there is less fat deposition (as lipid droplets) in the fish liver. Organic diet had 26.5% total plant meal while laboratory and conventional diet had 35.2% each. According to Carter, et al [18] the sea breams fed diets A, B and C showed very similar growth performance that was matched by indices of protein metabolism in their liver.

Most plant protein sources contain antinutritional factors that may affect growth, nutrient utilisation and fish welfare in general [20]. Hansen, et al [21] observed that cod can be fed diets with up to 440 g/kg plant protein ingredients. Small decreases in protein apparent digestibility coefficients (ADC) and larger decreases in fat ADC were observed with high levels of plant protein ingredients (corn gluten meal or soybean meal or wheat gluten meal). Robaiana, et al [22] found that the liver of *S. aurata* fed high levels of soybean meal showed higher lipid deposition. Our results agree with these results due to the less accumulation of lipid droplets into the liver when a higher percentage of fish meal was provided in the diet. Organic diet had no plant protein meals. The fish fed with an organic diet had a similar growth performance, despite the fact that the lipid content and thus the dietary energy content was lower in the diet in comparison to the commercial one.

Fat deposition in fish tissues is affected by many factors, including dietary lipid content, which has been positively related to the fat content in tissues [23,24]. The organic and laboratory diet used in the present study had lower lipid content than the commercial diet (14% vs. 17%). The lower dietary lipid content of the organic and laboratory diet probably resulted in the lower lipid content in sea bream's liver (smaller and lesser lipid droplets) in comparison with the group fed the commercial diet. Mente, et al [25] showed that the liver lipid content was lower and the hepatosomatic index was higher for the organic cultured *S. aurata*. Caballero, et al [16] using different lipid levels and high or low quality fish meal diets conclude that the hepatic morphology observed in *S. aurata* fed diets containing 22% lipid and high or low quality fish meal reflects the storage of lipidic reserve without pathological consequences. In addition when diets used with 27% lipid and high quality fish meal steatosis in the liver was observed that could reflect the non-utilization of dietary lipid. However, steatosis was not observed for diets with low quality fish meal and same lipid level. The above results show that the optimum balance between dietary lipid and protein level depends on the quality of the fish meal.

Berillis, et al [17] using the same diets (A, B and C) as in the present study conclude that diet can affect fish liver collagen fibril diameters since it contains some trace elements and vitamins that play an important role in collagen synthesis, metabolism, and fibril diameter. *S. aurata* fed with organic diet showed significantly bigger

collagen fibril diameters than fish fed the conventional diet. Furthermore, the organically fed fish had similar size collagen fibril diameters as the wild fish.

Acinar cells secrete the major enzymes involved in the digestion of foodstuffs. Many of these are secreted as inactive precursors. The enzymes or enzyme precursor proteins storage locus are the zymogen granules. The release of enzymes as inactive precursors ensures that the activated enzymes do not autodigest the pancreatic tissue. The concentration of enzymes in the zymogen granules vary with the dietary intake [26,27]. In actively feeding fish these contain large numbers of bright, eosinophilic, secretory zymogen granules. Digestive enzymes are secreted from these acinar cells into the anterior intestine to break down proteins, fats, and carbohydrates [9]. In freshwater teleosts, digestive enzyme activity is affected by feeding behaviour and biochemical composition of the food [28]. In our study, abundance and number similarity of zymogen granules in acinar cells, regardless of the used diet, suggest efficient food consumption, active production and secretion of pancreatic enzymes like protease, lipase, amylase esterase and phosphates indispensable for the digestion of macromolecules - protein, carbohydrates, and lipids/fats.

In summary, the organic diet produced similar zymogen granules and smaller lipid droplets with no signs of steatosis. The results of this study confirm that diets containing lower lipid amounts accumulate smaller lipid droplets into the *S. aurata* liver.

Authors' Contributions

Valaroutsou E. and Voudanta E. performed the tissue processing, examined the tissues under the photon microscope and analyzed the results. Mente E. contributed to the research protocol, was responsible for the fish sampling, contributed to the discussion of the results, writing and editing. Berillis P. deployed the research protocol, examined the tissues under the electron microscope, performed the image analysis, contributed to the analysis of the results, discussion and writing.

Conflict of Interest

The authors confirms that this article content has no conflicts of interest.

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