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ABSTRACT

This article proposes a systematic evaluation of methods pertaining to the pathology discipline (histology, histochemistry, ultrastructure, *in situ* techniques, image analysis including morphometry and fractal dimension analysis) employed to study liver and intestine status during a series of nutritional trials performed on flatfish species (common sole, *Solea solea*; Senegal sole, *Solea senegalensis* and turbot, *Scophthalmus maximus*) of commercial interest. Histology is the first step able to detect the normal architecture of the tissues or the possible occurring changes related to inflammation, degeneration and cell death. The evaluation of liver should include a scoring system to obtain semi-quantitative numerical data that are statistically analysable; histochemisty is a valid tool for identifying the type of cell content, together with ultrastructure that also permits the detection of even mild signs of cell damage. For the evaluation of the intestine, *in situ* techniques are useful together with fractal dimension analysis to determine the mucosal trophism, while ultrastructure can demonstrate cell injury. These considerations were resumed in a methodological flowchart as a valid tool to assess the fish health status when nutritional trials are planned.

Introduction

Fish histopathology has become an increasingly important discipline in fields that includes basic biomedical research, ecotoxicology, environmental resource management and aquaculture (Wolf et al. 2015). There is a wide range of experimental settings, from the pure toxicological trials where all the parameters are under strict control, to the ecotoxicological trials where wild individuals are randomly sampled. The aquaculture settings are in an intermediate level of parameters control (genetics of individuals, water parameters, nutrition). The histological diagnostic investigative methods can be potentially applied to all of these settings, and as a consequence, paraphysiological adaptations, pathological findings and changes can be compared.

In experimental nutritional trials, histopathology can provide additional information on the zootechnical performances to better explore mechanisms involved in digestion, feed utilisation, metabolism and

monitoring the overall health of reared fish. Histological analysis of the digestive system is considered a good indicator of the nutritional status of fish. The intestine and liver are the most important organs in metabolism, digestion and absorption of nutrients from food, and, therefore, monitoring the status of these organs is considered necessary (Rašković et al. 2011; Saraiva et al. 2016). Recently, a histological study as indicator of juveniles farmed turbot health status confirmed to be a useful tool for assessing the fish health (Saraiva et al. 2016).

The present work proposes a systematic evaluation of methods pertaining to the pathology discipline to study the liver and intestine status during a series of nutritional trials that were performed on flatfish species of commercial interest: common sole (*Solea solea*), Senegal sole (*Solea senegalensis*) and turbot (*Scophthalmus maximus*).

The methods proposed are arranged in a methodological flowchart and are subdivided into progressive

deep levels of investigations which have required increasing skills, costs and time, but which offer a univocal description and interpretation of the morphological changes that have occurred. This flowchart could be considered a valid tool when nutritional trials are planned.

Materials and methods

All the experimental trials were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with the European Community Council directive (86/609/ECC). The methods used have been broadly described in other publications from the present research group and are schematically represented in Tables 1 and 2, briefly presented and subdivided according to the two organs investigated and to the different nutritional trials.

Histology

Liver and intestine

Histology was performed in all the nutritional trials presented in this work. In all treatments, three fish per tank (9 per treatment) were sampled in triplicate at the end of 70–90 d nutritional trials.

Liver and intestine were sampled, fixed in 10% buffered formalin and processed for routine histology (H&E). The tissues were examined to evaluate degenerative and/or inflammatory changes (Gatta et al. 2011; Mandrioli et al. 2012).

Histochemistry

Liver

Histochemical stains were performed only in the research works in which the aim was to qualitatively characterise the cell content and, specifically, in flatfish fed isoenergetic diets with increasing protein levels (Gatta et al. 2011) and fed isoenergetic diets with variable protein/lipid levels (Mandrioli et al. 2012).

OilRedO: a sample of liver from two fish per tank (increasing protein levels, see Table 1 for details) was snap-frozen in liquid nitrogen. Quenching was accomplished by placing a small amount of embedding medium for frozen tissue specimens (OCT) (Tissue-Tek, Sakura Finetek, Torrance, CA) onto a cork disc where the sample was positioned, and then dropping it into a beaker containing isopentane and liquid nitrogen for 1 min and then storing at −80 °C. Frozen sections of 3 μm were cut in the cryostat (Leica Microsystems

Table 1. Summarizing table of methods and results for liver.

LIVER	Diets	Scoring system for lipid content within hepatocytes	Image analysis for morphometry	Histochemistry	Fractal dimension (FD) analysis	Ultrastructure (TEM)	Results
Solea solea fed increasing protein/lipid levels (Gatta et al. 2011)	Four isoenergetic diets with different crude protein levels: 39%, 45%, 51%, 57% dry weight	Semiquantitative: mild, moderate, severe fat accumulation (on H&E)	Not done	Oil Red O on frozen sections	Not done	Not done	Higher density of lipid droplets in high-protein/low-lipid diets; good demonstration of lipids with histochemistry
Senegalese sole fed variable protein/lipid levels (Mandrioli et al. 2012; Bianco et al. 2013)	Four isoenergetic diets with different protein and lipid levels (P/L, % dry matter: 44/22, 49/20, 54/18, 59/16)	Semiquantitative: from 2 to 4 according to the increased severity of fat accumulation (on H&E)	Empty area (EA), perimeter and roundness measured by ImageJ plugin segmentation (on H&E)	Toluidine blue on semi-thin sections	Box counting method (on H&E)	Ultrathin sections with uranyl acetate and lead citrate	Electrondense non-membrane bounded lipidic content; higher hepatocytes area and perimeter in diet with lower protein/higher lipid content. histological scoring correlate with empty area (EA) but not with fractal dimension (FD)

Table 2. Summarizing table of methods and results for intestine.

Intestine	Diets	Scoring system for lipid content within enterocytes	Image analysis for morphometry	Histochemistry	Fractal dimension (FD) analysis	Ultrastructure (TEM)	Results
Turbot fed plant proteins (Bonaldo et al. 2011)	Control diet with 52% crude protein, 18% crude fat and 25% plant protein inclusion level. Three diets isoproteic and isolipidic to the control with 39%, 52%, and 66% of plant protein by increasing the level of soybean meal, wheat gluten, and corn gluten	Not done	Not done	Not done	Not done	Not done	22.7% soybean meal did not exert any morphological change in the intestine; histology as good indicator of gut health
Solea solea fed different lipid levels (Bonvini et al. 2015)	Four isonitrogenous (59% protein) diets with different dietary lipid levels: 8%, 12%, 16%, 20%	Not done	Not done	Not done	Not done	Semi-thin sections with toluidine blue; ultrathin sections with uranyl acetate and lead citrate	Electrondense non-membrane bounded lipidic content and whorled myelin bodies (enterocytes overload) in diet with more than 12% lipids
Solea solea fed mussel meal (Sirri et al. 2014)	Four isoproteic (53%) and isolipidic (11%) diets containing graded levels of mussel meal 0%, 25%, 50%, 75% to replace fish meal	Not done	PCNA and TUNEL positivities measured by ImageJ plugin segmentation	Not done	Box counting method (on H&E)	Not done	Degeneration or inflammation were not present; PCNA index and FD decreased with high content of mussel meal

GmbH, Wetzlar, Germany) and stained with Oil red O (Gatta et al. 2011).

Toluidine Blue: small samples (about 1 mm³) of liver from one fish per diet (variable protein/lipid levels, see Table 1 for details) were fixed for 3 h with 5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2. After a thorough rinse in the same buffer, the samples were post-fixed for 1 h with 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2, dehydrated in a graded sequence of alcohols, and embedded in Durcupan AcM resin. Semi-thin sections were stained and examined by light microscopy to locate appropriate areas of the liver (Mandrioli et al. 2012).

Ultrastructure

Liver and intestine

The ultrastructural investigation (transmission electron microscopy, TEM) was included in one trial on a selection of subjects based on the histological evidence consistent with lipid accumulation. Once semi-thin sections were obtained, as described above for toluidine blue histochemistry, selected ultra-thin sections were cut from each specimen and stained with uranyl acetate and lead citrate. Ultrastructural observation was made with a Zeiss EM 109 transmission electron microscope (Carl Zeiss AG, Oberkochen, Germany) operated at 80 kV.

In the intestine, TEM was included in one trial in order to precisely localise the lipid accumulation and the possible signs of cell damage; the ultrastructural observation was made with a Philips 208 TEM (Philips, Eindhoven, Holland) operating at 100 kV (Mandrioli et al. 2012; Bonvini et al. 2015).

In situ *techniques*

The *in situ* techniques (anti-PCNA immunohistochemistry and TUNEL method) were used to assess cellular turnover of enterocytes in flatfish fed mussel meal diets (Sirri et al. 2014) (Table 2).

Anti-proliferating cell nuclear antigen (PCNA) antibody was employed for the detection of the proliferation rate of enterocytes. Sections were deparaffinized for 30 min in Solvent Plus (Carlo Erba, Reagents S.r.l., Cornaredo, Italy) and hydrated in a graded series of alcohols. Subsequently, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 30 min at room temperature. Sections were then treated in citrate buffer (pH 6.0) for 10 min in a microwave oven (750 W) for antigen retrieval. Non-specific binding was blocked by incubating sections

with 5% normal goat serum and 1% bovine serum albumin (BSA) in PBS buffer (blocking solution) for 1 h at room temperature and then sections were incubated overnight at 4 °C with mouse monoclonal PCNA antibody (clone PC10, Cell Signaling Technology, Danvers, MA) diluted 1:4000 in blocking solution. A streptavidin–biotin–peroxidase polymeric complex (Super-Picture kit peroxidise, Zymed® Lab, San Francisco, CA) was used to visualise antibody–antigen interaction and applied on the sections for 10 min at room temperature. The immunohistochemical reaction was developed using a 3,3′-diaminobenzidine (DAB) solution (Sigma-Aldrich Corporate, St. Louis, MO). The slides were counterstained with haematoxylin, dehydrated through alcohol and cleared in xylene before mounting.

The TUNEL method was used to detect the apoptosis rate of enterocytes. The ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore Corporation, Billerica, MA) was employed. Sections were deparaffinized for 30 min in Solvent Plus (Carlo Erba Reagents S.r.l., Cornaredo, Italy) and hydrated in a graded series of alcohols. Treatment with proteinase K (20 μg/ml) in PBS for 15 min was performed. Subsequently, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 5 min at room temperature. Equilibration buffer was then applied on the sections for 3 min and finally, they were incubated with working strength TdT enzyme for 1 h at 37 °C in a humid chamber. After washing with stop/wash buffer for 10 min, the secondary antibody anti-digoxigenin peroxidase conjugate was applied on the sections for 30 min at room temperature. After washing, the sections were subjected to the same protocol as used for PCNA.

Image analysis

Liver

Scoring system. Coded histological sections of liver were scanned at 40× with a light microscope and 10 random fields were selected; according to the severity and the distribution pattern of the hepatic lipidic storage/infiltration, a semiquantitative scoring system of four classes was used for lipidic infiltration assessment (1 = normal; 2 = mild; 3 = moderate; 4 = severe) (Gatta et al. 2011; Mandrioli et al. 2012).

Morphometry. Two different methods of image analysis were applied to calculate the percentage of hepatic area occupied by lipid droplets (Mandrioli et al. 2012; Bianco et al. 2013). (1) Five fields of each liver

section were randomly selected and blindly evaluated with respect to group, resulting in a total of 180 fields. Digital images of the selected fields were taken with a Leica DMLB (Solms, Germany) microscope on which a Leica DFC320 (Solms, Germany) digital camera was mounted. The images were acquired at a 630× magnification and at 24-bit, saved in TIFF format; each selected field had a similar area of 2.1×10^{-2} mm^2. The ImageJ software (Wayne Rasband, NH, Bethesda, MD, http://imagej.nih.gov/ij/) was used for image analysis; a standard grid with four horizontal and five vertical lines to obtain 20 cross points was set up and superimposed on each image. Only the hepatocytes lying on each cross point were considered. Each hepatocyte was further identified by the device, marking the entire perimeter of its cytoplasmic border. The section area, perimeter and roundness were automatically measured by the software. A total of 3600 hepatocytes were measured (100 for each liver).

(2) Digital image analysis was performed by ImageJ 1.46 plugin segmentation (http://bigwww.epfl.ch/sage/soft/colorsegmentation/). Each image was manually segmented and partitioned in two pixel clusters, the area involved by lipid accumulation (empty area = EA) and the remaining area, both expressed as a percentage.

Intestine

Cell counting. Cell counting was performed using the plugin colour segmentation of ImageJ software (http://rsbweb.nih.gov/ij/plugins/index.html#segmentation) for PCNA-immunopositive and TUNEL-positive enterocytes (Sirri et al. 2014). From each stained section, photomicrographs of five fields per gastrointestinal tract at 20 × magnification were captured using a Nikon Digital Sight SD-MS camera (Nikon Corporation, Tokyo, Japan) connected to an optical microscope. Images were then processed using ImageJ 1.46 software (Wayne Rasband, NH, Bethesda, MD), which is freely downloadable. Acquired photographs of PCNA- and TUNEL-stained slides were in 24- bit jpg format, with an image resolution of 2560 × 1920 pixels. PCNA-positive nuclei of enterocytes were counted using the plugin colour segmentation. The original image is divided into colour channels, which are manually chosen by the operator as DAB-stained positive nuclei, haematoxylin-stained negative nuclei, cytoplasm of enterocytes, and white background. The software automatically calculates the percentage of area occupied by each chosen colour and, specifically, the percentage of area occupied by PCNA-positive (DAB-stained) nuclei. In order to eliminate false

positivity of non-epithelial cells (erythrocytes, macrophages, lymphocytes and fibroblasts), the lamina propria had been manually erased using the software Adobe Photoshop CS5 (Adobe Systems Inc, San Jose, CA). For apoptotic cell count, the following findings were considered to represent apoptosis: (a) marked condensation of chromatin and cytoplasm (apoptotic cells); (b) cytoplasmic fragments with or without condensed chromatin (apoptotic bodies); and (c) intracellular and extracellular chromatin (apoptotic micronuclei). TUNEL-positive cells were counted manually in order to eliminate the non-specific signals due to the application of this method on intestinal sections, and the plugin colour segmentation was employed only to calculate the percentage of area occupied by the other colours (haematoxylin-stained negative nuclei, cytoplasm of enterocytes and white background). All data obtained were used to calculate PCNA and apoptotic indexes.

Liver and intestine

Fractal dimension analysis. Box-counting fractal dimension (FD) analysis was performed to assess the lipid storage severity and the accumulation pattern (Bianco et al. 2013). Images taken from H&E stained slides were subjected to a segmentation process and minimally manipulated to obtain the outline of the lipid droplets. Contrast was enhanced to emphasise the difference between lipid droplets and the remaining parenchyma, and then binarisation was performed. Computing of FD was performed with the box counting method with ImageJ 1.46 (http://rsb.info.nih.gov/ij/) using boxes of 2, 3, 4, 6, 8, 12, 16, 32, 64, 128, 256, 512, 1024 and 2048 pixels. Calculation of FD takes approximately 10 min for 180 photographs.

In the intestine, FD analysis was performed to assess diet-induced hyperplastic changes of the mucosa (Sirri et al. 2014). Eighty photomicrographs of H&E-stained slides of intestinal tracts were obtained in a blinded fashion at 4 × magnification using a Nikon Digital Sight SD-MS camera (Nikon Corporation, Tokyo, Japan) connected to an optical microscope. Images were then processed using ImageJ 1.46 software (Wayne Rasband, NH, Bethesda, MD). Manual segmentation and thresholding were performed blindly by a second operator to extract the one-pixel wide outline of the intestinal mucosal interface (outline). The FD calculation was performed with the box counting method using the specific command in ImageJ and setting boxes of 2, 3, 4, 6, 8, 1216, 32, 64, 128, 256, 512, 1024 and 2048 pixels. Image resolution was 2560 × 1920 pixels and the format was 24 bit TIF for the original acquired images of H&E-stained slides;
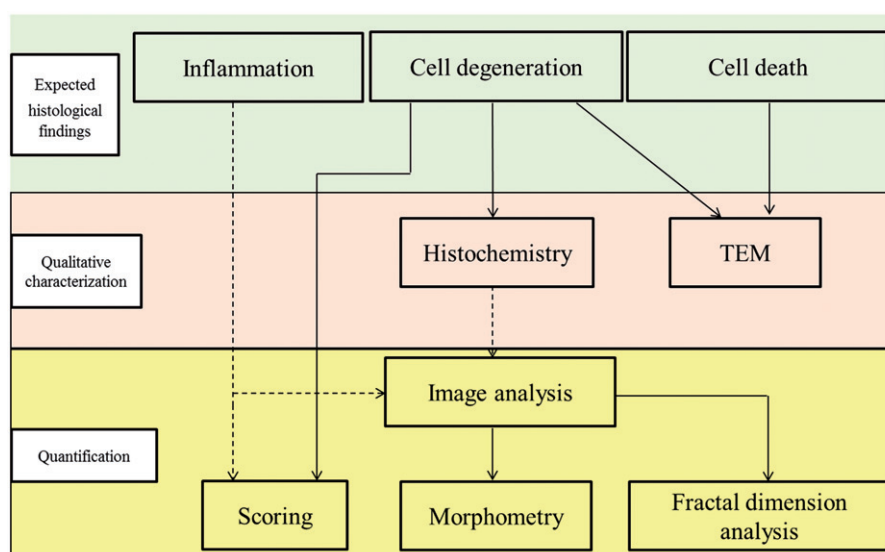
Figure 1. Colour-coded flowchart of methods employed for liver. Subsequent levels of methodological deepening adopted. Solid lines indicate the steps employed in this work. Dashed lines indicate other possible way of application and not used in this work.

image resolution was 2560 × 1920 pixels and the format was 8 bit Gif for the acquired photographs of outlines.

Results and discussion

In Figures 1 and 2, are represented the methodological colour coded flowcharts for liver and intestine as results of interrelation among the histological techniques used. Figures 3 and 4 show an overview of the methods employed for liver and intestine, respectively.

Histology

On the basis of our studies on flatfish, histological analysis, even though may be considered the basic step, confirmed to be a valid and fundamental tool permitting to evaluate the health status of hepatic and intestinal tissues. Similarly, other studies that have included histological analyses complementary to tissue composition and blood chemistry, did not reveal the existence of any intestinal alterations induced by the inclusion of different plant proteins in Senegal sole juveniles (Rodiles et al. 2015). Saraiva et al. (2016) performed an histological study conducted in an intensive farm of juveniles turbot concluding that this is a useful tool for assessing the fish health and demonstrating that most of the fish were in good health condition.

Histopathology remains one of the most reliable, sensitive and comprehensive assays for identifying and characterising a vast array of physical disorders (Wolf et al. 2015). In this support, several papers reported hepatic degenerative changes such as formation of lipid vacuoles, pyknosis, cell necrosis or adaptive changes such as hypertrophy and, more rarely, haemorrhages in various fish species in case of overfeeding or when they were fed vegetable and animal oils, ginger peel meal, mannan oligosaccharides (Parpoura and Alexis 2001; Taddese et al. 2014).

About intestinal changes, turbot fed high level of plant protein showed decreased height and increased fusion of the mucosal folds, increased width and cellular infiltration of the lamina propria and submucosa, reduced numbers of supranuclear absorptive vacuoles and displaced nuclei toward the apexes of the cells, interpreted as soy-bean-meal-induced enteritis changes (Gu et al. 2017). The compartimentalized supranuclear vacuoles are an interesting histological feature: they can vary in size depending on the particle size of the ingested material. The contained intravacuolar particles are then directed to intraepithelial macrophages for phagocytosis and processing. Specific changes can occur to supranuclear vacuoles during inflammatory events, including a decrease in size and gradual disappearance that has been noted in cases of soybean meal enteritis, with a corresponding reduction in endocytosis that resolves once the inciting antigen (i.e. soy saponin) has been removed from the diet (Peterson 2015).

Histochemistry

Histochemistry has been confirmed to be a valid tool helping to identify the type of cell content; however, due to specificity of the results, it can be employed only when moderate to severe content accumulation
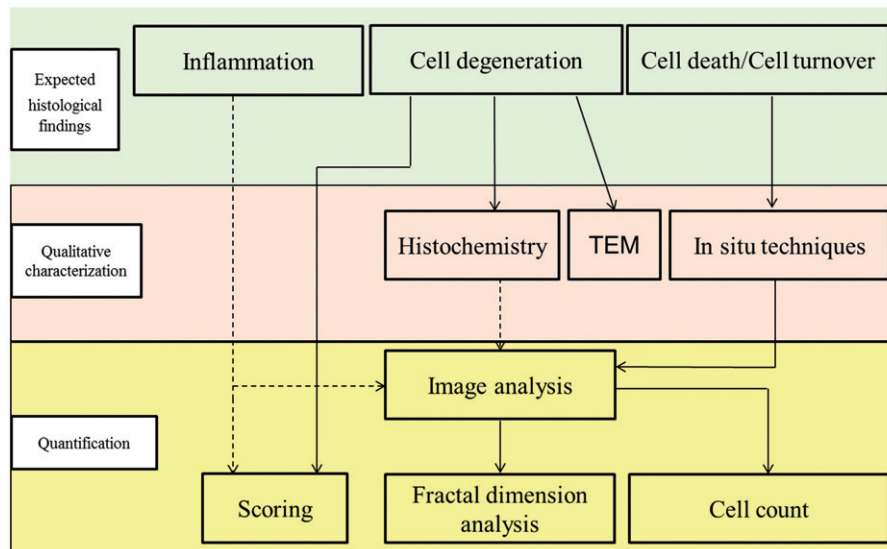
Figure 2. Colour-coded flowchart of methods employed for intestine. Subsequent levels of methodological deepening adopted. Solid lines represent the steps employed in this work. Dashed lines indicate other possible way of application and not used in this work.
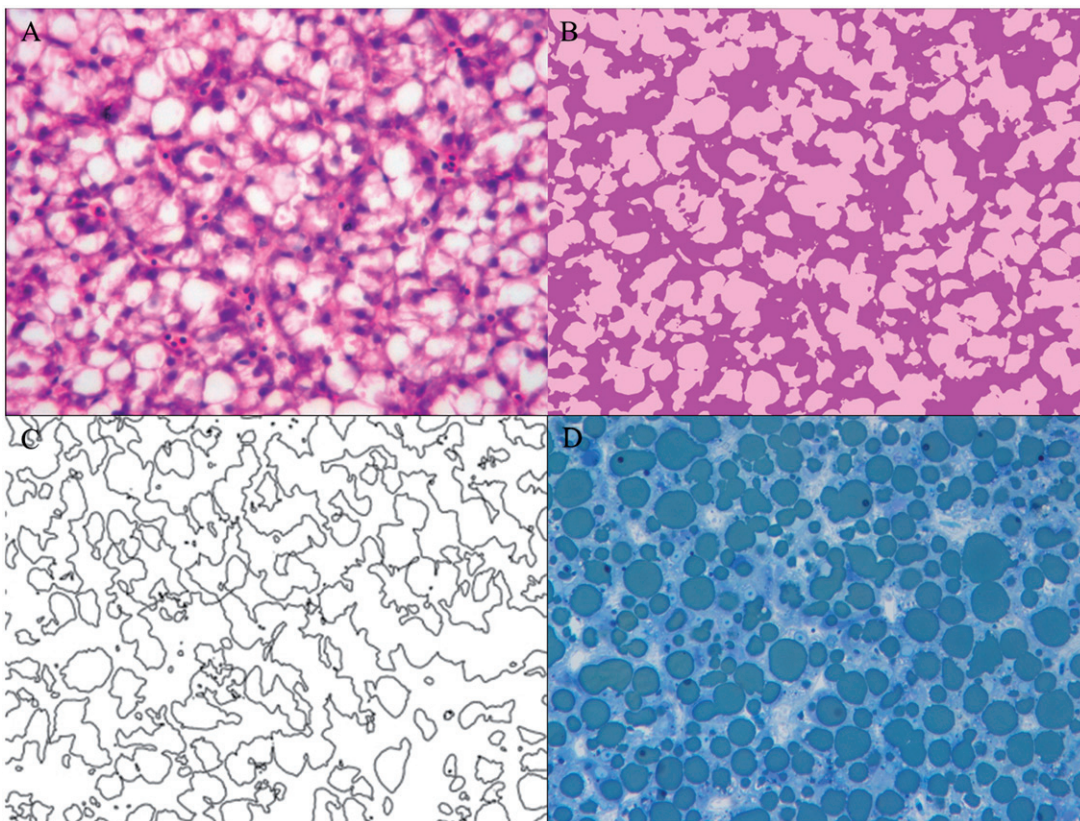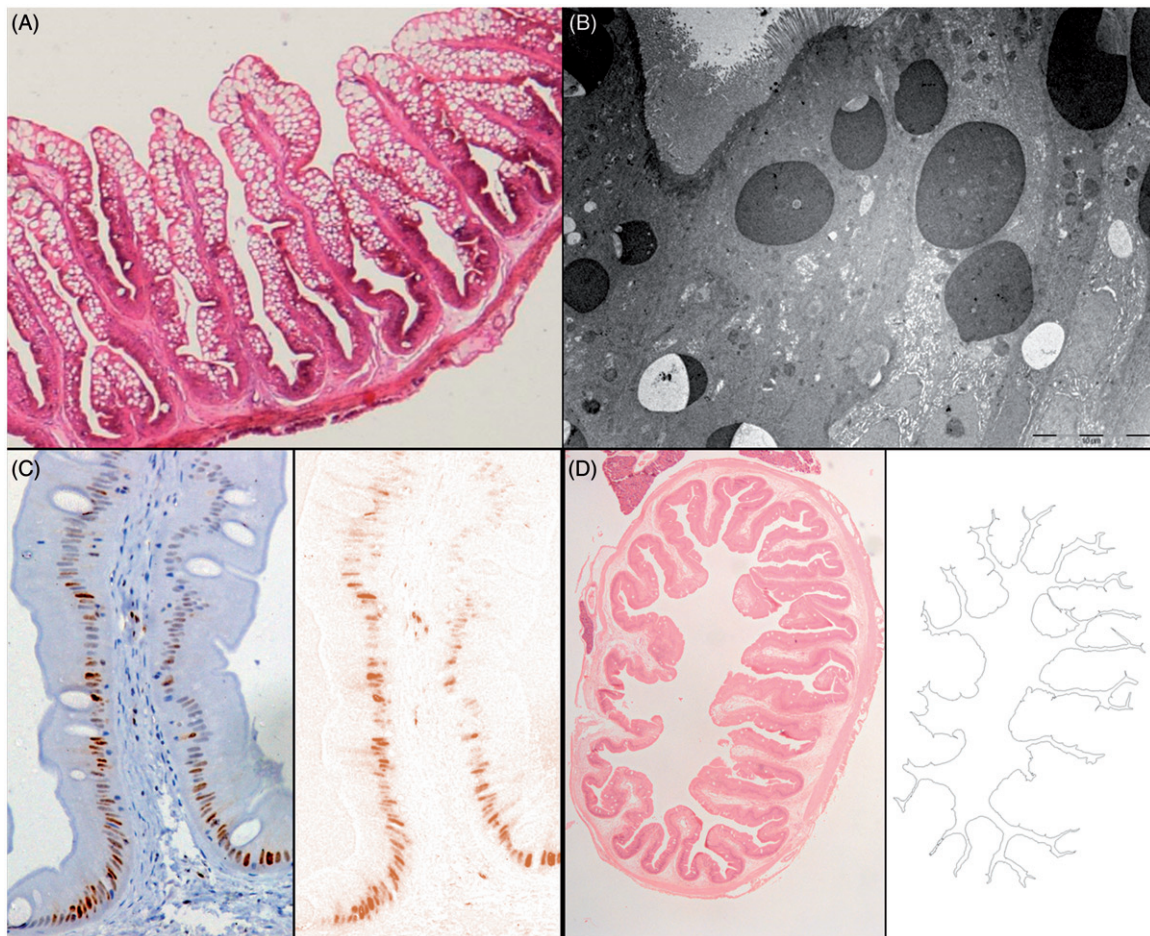

Figure 3. Example of methods employed for liver. (A) Histological section of a moderate hepatic lipid accumulation; (B) the same section processed by image analysis to obtain the percentage of area involved in the lipid accumulation; (C) subsequent image analysis processing to obtain hepatocytes' profile for fractal dimension analysis; (D) histochemistry with toluidine blue on a semi-thin section demonstrating grey-blue lipid droplets.

is the expected result. In the study reported by Gatta et al. (2011), OilRedO stain was used to detect lipid content accumulation in the liver, based on the greater solubility of the dye in neutral fats than in the solvent in which it is dissolved. More commonly other stains as PAS are used to detect different substrates that can be accumulated in the cells as glycogen in the liver (Rodiles et al. 2015).

Figure 4. Example of methods employed for intestine. (A) Histological section of intestinal steatosis; (B) ultrastructure of the same case showing large electrondense lipid droplets; (C) immunohistochemistry with anti-PCNA antibody detecting proliferating enterocytes' nuclei and the corresponding image analysis processing for cell count; (D) histological transversal section of intestine processed for fractal dimension analysis mucosal profile.

Among our trials, toluidine blue was performed only in the paper by Mandrioli et al. (2012) as an intermediate step in the ultrastructure processing technique. This stain is applied on semi-thin sections of samples routinely processed for TEM and embedded in resin and can allow the detection of lipid droplets within the cells (Bradford et al. 2010; Valaroutsou et al. 2013).

Ultrastructure

It is considered the gold standard and was confirmed as the best technique to detect even mild signs of cell damage and to localise the lipid content. According to the score of intracytoplasmic lipid accumulation presented by Mandrioli et al. (2012) in common sole liver, the ultrastructure examination showed multiple small or single large lipid electrondense droplets that progressively pulled the nucleus to the cell periphery but without revealing signs of subcellular suffering.

These findings were interpreted as paraphysiologic adaptive changes considering that Pleuronectidae tend to accumulate dietary lipids in the liver (Akiyoshi et al. 2001). Similar results have also been reported in gilthead seabream (*Sparus aurata*) fed dietary vegetable oils (Wassef et al. 2007). On the other hand, in the same species fed different dietary lipid levels combined with fish meal quality, Caballero et al. (1999) interpreted similar observations as a pathological process.

In a study on common sole, the ultrastructure of intestine displayed a cellular engulfment due to lipid overload; large electrondense lipid droplets (triglycerides) were found in the cytoplasm of enterocytes and in the Golgi apparatus, while myelinosomes (stacked phospholipids) were found in lysosomes (Bonvini et al. 2015). Caballero et al. (2003) employed ultrastructure for the study of intestine in gilthead seabream fed diets containing different lipid sources; an accumulation of supranuclear lipid droplets and the formation

of lipoproteins was observed with a proportionally increased percentage of fish oil replaced. Also in turbot, TEM was successfully employed to describe the subtle pathological changes occurring into intestinal microvilli of fish fed a diet with 40% soybean meal (SBM) compared with a diet with SBM and supplemented with 0.2% mannan oligosaccharide and to a standard diet without SBM (Bai et al. 2017).

The limitations of the use of the ultrastructural technique are mainly due to the costs and needing of specialised instruments and technicians that do not permit to consider TEM as a routine method.

Image analysis

Scoring system

A practical and simple scoring system consists in the adoption of a progressive numeric scale, attributing a number in relation to the severity of the process. It requires the knowledge of the paraphysiologic status of the tissue examined. In general it can be used to have preliminary information about the status and can be accompanied by a brief histological description of the slide.

Scoring systems of hepatic accumulation have been used in different fish species fed dietary inclusion such as vegetable oils, plant meal and soya-saponin, medical herbs, and functional feeds (Mourente et al. 2005; Martinez-Rubio et al. 2013; Rodiles et al. 2015). The strength of the scoring system is the use of semi-quantitative data classes that could be compared by statistical analysis.

For intestine, scoring systems have been recently used in turbot fed different SBM-based diets, detecting pathological changes related to a soy-induced enteropathy (Gu et al. 2016; Bai et al. 2017).

Morphometry

Morphometry consists of the use of quantitative data in the description of structural features, which can be obtained by various means (Reid 1980). It detects structural and morphological changes and provides accuracy to support the diagnostic decision. The morphometric approach includes item classification and grading, point counting and intersection counting methods, and the use of various semiautomatic or automatic instruments.

The data obtained by Mandrioli et al. (2012), in Senegal sole juveniles confirm that an excessive dietary lipid content leads to a massive storage of unused lipid within hepatocytes, as disclosed by the morphometric evaluation showing differences among diet groups in hepatocyte area and perimeter. These findings were interpreted as paraphysiological adaptive changes of the liver (Mandrioli et al. 2012). The same images were also analysed using a purely quantitative method that consisted of segmentation partitioning of images in an area occupied by lipids (optically empty area, EA) and the remaining parenchyma (Bianco et al. 2013). Escaffre et al. (2007) used morphometric procedures such as hepatocyte mean volume and nuclear mean diameter to evaluate cytoplasmic accumulation in rainbow trout (*Oncorhynchus mykiss*) fed soy protein concentrate, while Zakes et al. (2008) employed similar parameters for studying the effects of two medicinal herbs on the growth performance of juvenile pike-perch (*Sander lucioperca* L.). In Senegal sole-fed plant proteins, the area of hepatocytes was measured by Rodiles et al. (2015).

Intestinal and liver morphometry was applied in yellow tail tetra (*Astyanax altiparanae*) fed with oregano oil by measuring folds height and width, muscle layer thickness and a superimposed grid on images of liver aiming to calculate the core percentages of the cytoplasm and capillaries (Ferreira et al. 2016).

Integrative approach of histopathology and histomorphometry as marker of general fish health state in pond culture was performed on common carp (*Cyprinus carpio* L.) organs (Rašković et al. 2016). In that study, the histomorphometric results showed larger cytoplasmic areas of hepatocytes in fish fed grain cereals, which was attributed to glycogen accumulation and higher numbers of goblet cells in the distal intestines of fish fed compound feed.

In turbot fed diets supplemented with stachyose or fed SBM-based diets supplemented with nucleotides, morphometry has been used to measure fold height, enterocytes height, microvillus height to quantify pathological changes (Peng et al. 2013; Hu et al. 2015).

In addition, morphometry by image analysis was employed in turbot fed diets with inclusion of soya allergens, β-conglycinin and glicynin, as antinutritional factors to determine the perimeter ratio (PR) of the intestine lumen as absorptive surface area (Li et al. 2016, 2017).

Unfortunately, regarding morphometry, several different methodological approaches have been used in fish feeding trials, making almost impossible the comparison between them. Image analysis by segmentation has been used on common sole for quantification of PCNA- and TUNEL-positive enterocytes (markers of proliferation and apoptosis, respectively) (Sirri et al. 2014). In this study the classic evaluation of cell turnover has been transposed from mammals to fish since hyperplasia of mucosal folds was an expected result in

relation to a specific dietary ingredient. Regarding enterocytes proliferation, in fish, the Ki67 antigen cannot be labelled with the specific MIB-1 antibody, which does not cross-react. Thus, MIB-1 antibody has been classically replaced with PCNA antibody and evaluated for better understanding the homeostatic response of fish intestinal epithelium to diet components (nutritional stress) (Ostaszewska et al. 2008).

Fractal dimension analysis

Fractal geometry provides formal mathematical foundations to better understand and characterise a large number of phenomena where structural complexity, irregularity and seemingly random morphology are a common motif (Landini 2011); it is widely applied in biomedical sciences, from diagnostic imaging to histopathological fields (Tambasco and Magliocco 2008; Landini 2011). To the best of our knowledge, in fish tissues, only two studies have been performed using FD analysis (Bianco et al. 2013; Sirri et al. 2014). In the first one, FD analysis of liver texture given by the lipid storage pattern (microvesicular or macrovesicular) was compared with the area of lipid accumulation (empty area, EA) in sole. Unexpectedly, a correlation between FD and semi-quantitative scoring and between FD and EA was not present, while the scoring was strongly correlated to EA. In this view, the segmentation of histological images of liver was proposed as a robust method in assisting the histological scoring, and the percentage value for EA of 55% was identified as the cut-off between severe (over 55%) and mild-to-moderate hepatic lipid accumulation (below 55%). FD can be useful, but cannot be considered the best method in interpreting the progression of lipid accumulation in the fish liver while the measure of area (EA) involved by lipid content showed higher values of sensitivity and specificity when compared with the histological scoring.

Sirri et al. (2014) demonstrate that FD analysis is a valid tool to quantify the adaptive response of intestinal mucosa of common sole. The FD calculation is able to transduce shape complexity of mucosal folds in analytical quantitative data, harmonising the gap between structural features (typically qualitative) and functional quantitative measures. The results obtained in the above-mentioned paper demonstrated a relationship between reduced enterocytes proliferation and lessened complexity of mucosal folding, expressed by a lower FD. This study demonstrated this usefulness calculation as an alternative method to *in situ* techniques for the detection of intestinal trophism.

Conclusions

In conclusion, a retrospective analysis of histological and other techniques pertaining to pathology employed in experimental nutritional trials of flatfish species was performed. An histology-based flowchart method is proposed to identify degenerative or adaptive changes of the liver and intestine in relation to the diet. Histology is the first step able to detect the normal architecture of the tissues or the possible occurring changes related to inflammation, degeneration and cell death. The scoring system is employed to quantify the severity of tissue degeneration and inflammation. Histochemistry is employed to characterise the nature of cell content in case of tissue degeneration and TEM contribute in identifying signs of cell suffering. Image analyses that include morphometry and fractal dimension analyses provide a quantification of cell degeneration and/or adaptive changes of the intestinal mucosal folds. The *in situ* techniques (anti-PCNA immunohistochemistry and TUNEL method) are used to assess cell turnover. The use of this flowchart can support experimental designing to identify the most appropriate methodological approach aimed to assess fish health under nutritional trials. The methods proposed in this study on flatfish can also be applied to other teleost species. Since the literature reports a high variability of histological/morphometrical methods employed to assess fish health during nutritional trials, this study may help to standardise the most appropriate techniques facilitating comparison among researches.

Disclosure statement

The authors declare no competing interests.

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References

Akiyoshi H, Inoue A, Hamana A. 2001. Comparative histochemical studies of the livers of marine fishes in relation to their behavior. Bull Fac Life Environ Sci Shimane Univ. 6:7–16.

Bai N, Gu M, Xu X, Krogdahl Å. 2017. Protective effects of mannan oligosaccharides on turbot *Scophthalmus maximus* suffering from soy enteropathy. Aquaculture. 476: 141–151.

Bianco C, Sirri R, Sarli G, Ostanello F, Bonaldo A, De Vico G, Mandrioli L. 2013. How severe is the lipid accumulation in fish liver? A fractal answer. Fractal Lab J. 2:3.

Bonaldo A, Parma L, Mandrioli L, Sirri R, Fontanillas R, Badiani A, Gatta PP. 2011. Increasing dietary plant proteins affects growth performance and ammonia excretion but not digestibility and gut histology in turbot (*Psetta maxima*) juveniles. Aquaculture. 318:101–108.

Bonvini E, Parma L, Mandrioli L, Sirri R, Brachelente C, Mongile F, Gatta PP, Bonaldo A. 2015. Feeding common sole (*Solea solea*) juveniles with increasing dietary lipid levels affects growth, feed utilization and gut health. Aquaculture. 449:87–93.

Bradford EM, Miller ML, Prasad V, Nieman ML, Gawenis LR, Berryman M, Lorenz JN, Tso P, Shull GE. 2010. CLIC5 mutant mice are resistant to diet-induced obesity and exhibit gastric hemorrhaging and increased susceptibility to torpor. Am J Physiol Regul Integr Comp Physiol. 298:R1531–R1542.

Caballero MJ, Izquierdo MS, Kjorsvik E, Montero D, Socorro J, Fernandez AJ, Rosenlund G. 2003. Morphological aspects of intestinal cells from gilthead seabream (*Sparus aurata*) fed diets containing different lipid sources. Aquaculture. 225:325–340.

Caballero MJ, Lopez-Calero G, Socorro J, Roo FJ, Izquierdo MS, Fernandez AJ. 1999. Combined effect of lipid level and fish meal quality on liver histology of gilthead seabream (*Sparus aurata*). Aquaculture. 179:277–290.

Escaffre AM, Kaushik S, Mambrini M. 2007. Morphometric evaluation of changes in the digestive tract of rainbow trout (*Oncorhynchus mykiss*) due to fish meal replacement with soy protein concentrate. Aquaculture. 273:127–138.

Ferreira PMF, Caldas DW, Salaro AL, Sartori SSR, Oliveira JM, Cardoso AJS, Zuanon JAS. 2016. Intestinal and liver morphometry of the Yellow Tail Tetra (*Astyanax altiparanae*) fed with oregano oil. An Acad Bras De Cienc. 88:911–922.

Gatta PP, Parma L, Guarniero I, Mandrioli L, Sirri R, Fontanillas R, Bonaldo A. 2011. Growth, feed utilization and liver histology of juvenile common sole (*Solea solea* L.) fed isoenergetic diets with increasing protein levels. Aquacult Res. 42:313–321.

Gu M, Bai N, Kortner TM. 2017. Taurocholate supplementation attenuates the changes in growth performance, feed utilization, lipid digestion, liver abnormality and sterol metabolism in turbot (*Scophthalmus maximus*) fed high level of plant protein. Aquaculture. 468:597–604.

Gu M, Bai N, Zhang Y, Krogdahl Å. 2016. Soybean meal induces enteritis in turbot *Scophthalmus maximus* at high supplementation levels. Aquaculture. 464:286–295.

Hu H, Zhang Y, Mai K, Ai Q, Xu W, Zhang W, Li Y, Liu J. 2015. Effects of dietary stachyose on growth performance, digestive enzyme activities and intestinal morphology of juvenile turbot (*Scophthalmus maximus* L). J Ocean Univ China. 14:905–912.

Landini G. 2011. Fractals in microscopy. J Microsc. 241:1–8.

Li Y, Hu H, Liu J, Yang P, Zhang Y, Ai Q, Xu W, Zhang W, Mai K. 2016. Dietary soya allergen β-conglycinin induces intestinal inflammatory reactions, serum-specific antibody response and growth reduction in a carnivorous fish species, turbot *Scophthalmus maximus* L. Aquacult Res. 1–16.

Li Y, Yang P, Zhang Y, Ai Q, Xu W, Zhang W, Zhang Y, Hu H, Liu J, Mai K. 2017. Effects of dietary glycinin on the growth performance, digestion, intestinal morphology and bacterial community of juvenile turbot, *Scophthalmus maximus* L. Aquaculture. 479:125–133.

Mandrioli L, Sirri R, Gatta PP, Morandi F, Sarli G, Parma L, Fontanillas R, Bonaldo A. 2012. Histomorphologic hepatic features and growth performances of juvenile Senegalese sole (*Solea senegalensis*) fed isoenergetic practical diets with variable protein/lipid levels. J Appl Ichthyol. 28:628–632.

Martinez-Rubio L, Morais S, Evensen Ø, Wadsworth S, Vecino JG, Ruohonen K, Bell JG, Tocher DR. 2013. Effect of functional feeds on fatty acid and eicosanoid metabolism in liver and head kidney of Atlantic salmon (*Salmo salar* L.) with experimentally induced heart and skeletal muscle inflammation. Fish Shellfish Immunol. 34:1533–1545.

Mourente G, Good JE, Bell JG. 2005. Partial substitution of fish oil with rapeseed, linseed and olive oils in diets for European sea bass (*Dicentrarchus labrax* L.): effects on flesh fatty acid composition, plasma prostaglandins E2 and F2a, immune function and effectiveness of a fish oil finishing diet. Aquacult Nutr. 11:25–40.

Ostaszewska T, Dabrowski K, Hliwa P, Gomolka P, Kwasek K. 2008. Nutritional regulation of intestine morphology in larval cyprinid fish, silver bream (*Vimba vimba*). Aquacult Res. 39:1268–1278.

Parpoura AA, Alexis MN. 2001. Effects of different dietary oils in sea bass (*Dicentrarchus labrax*) nutrition. Aquacult Int. 9:463–476.

Peng M, Xu W, Ai Q, Mai K, Liufu Z, Zhang K. 2013. Effects of nucleotide supplementation on growth, immune responses and intestinal morphology in juvenile turbot fed diets with graded levels of soybean meal (*Scophthalmus maximus* L.). Aquaculture. 392:51–58.

Peterson TS. 2015. Overview of mucosal structure and function in teleost fishes. In: Beck BH, Peatman E, editors. Mucosal health in aquaculture. 1st ed. Waltham (MA): Academic Press; p. 55–65.

Rašković BS, Ciric M, Koko V, Stanković MB, Zivic I, Marković Z, Poleksić V. 2016. Effect of supplemental feeds on liver and intestine of common carp (*Cyprinus carpio*) in semi-intensive rearing system: histological implications. Biologia (Poland). 71:212–219.

Rašković BS, Stanković MB, Marković ZZ, Poleksić VD. 2011. Histological methods in the assessment of different feed effects on liver and intestine of fish. J Agric Sci. 56:87–100.

Reid IM. 1980. Morphometric methods in veterinary pathology: a review. Vet Pathol. 17:522–543.

Rodiles A, Herrera M, Hachero-Cruzado I, Ruiz-Jarabo I, Mancera JM, Cordero ML, Lall SP, Alarcòn FJ. 2015. Tissue composition, blood biochemistry and histology of digestive organs in Senegalese sole (*Solea senegalensis*) juveniles fed diets containing different plant protein ingredients. Aquacult Nutr. 21:767–779.

Saraiva A, Costa J, Eiras JC, Cruz C. 2016. Histological study as indicator of juveniles farmed turbot, *Scophthalmus maximus* L. health status. Aquaculture. 459:210–215.

Sirri R, Bianco C, De Vico G, Carella F, Bonaldo A, Sarli G, Tondini G, Mandrioli L. 2014. Proliferation, apoptosis, and

fractal dimension analysis for the quantification of intestinal trophism in sole (*Solea solea*) fed mussel meal diets. BMC Vet Res. 10:148.

Taddese F, Huh MD, Bai SC. Vijverberg. 2014. Histological changes of liver in overfed young Nile tilapia. J Fish Aquat Sci. 9:63–74.

Tambasco M, Magliocco AM. 2008. Relationship between tumor grade and computed architectural complexity in breast cancer specimens. Hum Pathol. 39:740–746.

Valaroutsou E, Voudanta E, Mente E, Berillis P. 2013. A microscopy and image analysis study of the liver and exocrine pancreas of sea bream *Sparus aurata* fed different diets. Int J Zool Res. 3:54–58.

Wassef EA, Wahby OM, Sakr EM. 2007. Effect of dietary vegetable oils on health and liver histology of gilthead seabream (*Sparus aurata*) growers. Aquacult Res. 38:852–861.

Wolf JC, Baumgartner WA, Blazer VS, Camus AC, Engelhardt JA, Fournie JW, Frasca S Jr, Groman DB, Kent ML, Khoo LH, et al. 2015. Nonlesions, misdiagnoses, missed diagnoses, and other interpretive challenges in fish histopathology studies: a guide for investigators, authors, reviewers, and readers. Toxicol Pathol. 43:297–325.

Zakes Z, Kowalska A, Demska-Zakes K, Jeney G, Jeney Z. 2008. Effect of two medicinal herbs (*Astragalus radix* and *Lonicera japonica*) on the growth performance and body composition of juvenile pikeperch [*Sander lucioperca* (L.)]. Aquacult Res. 39:1149–1160.