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Multiparametric Semi-quantitative Scoring System for the histological evaluation of marine fish larval and juvenile quality

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ABSTRACT

Gilthead seabream (GSB - *Sparus aurata*) and European seabass (ESB - *Dicentrarchus labrax*) are two of the most farmed fish species in EU. However, production of sea bream/bass in the EU has remained stagnant for the last decade and the Mediterranean EU aquaculture faces significant sustainability challenges. In consideration of this, and as it is largely recognized that the success of marine aquaculture strictly depends on the production of good quality larvae/juveniles, in this paper the authors put forward an original standardized tool for the histological assessment of GSB and ESB larva/juveniles. This tool promptly allows to highlight problems in marine fish larval batches because of managerial practices, suggesting to fish farmers which direction take to resolve them. A Multiparametric Semi-quantitative Scoring System (scoring range 1–5) has been originally developed for larval/juvenile histological evaluation and it includes 18 descriptors related to 6 organ districts. The values of each descriptor can be summarized in two indexes: the CHI (Cumulative Histological Index), giving general information about the quality of a fish batch in that precise moment and the OCV (Organ condition value) showing the general condition of each organ and by the individual descriptors. The paper purposes are to describe the MSSS, the criteria established for the score attribution and to supply some indications for the use of the tool.

1. Introduction

According to the last FAO data, 30.8 million tons (USD 106.5 billion) of aquatic animals have been produced from mariculture and coastal aquaculture combined in 2018; about 23 % (7.3 million tons) of this production is constituted by finfish (FAO, 2020) and this value is constantly growing over the past years (FAO, 2016). Increasing production requires increasing fingerling supply; it is now largely recognized that the success of mariculture strictly depends on the production of good quality larvae/juveniles. Nevertheless, the hatchery phase still remains one of the main bottlenecks of this sector. The high mortality (often more than 80 %) in the first weeks after hatching remains a challenging problem that needs to be solved (Vadstein et al., 2013). Although the increased knowledge on fish biology and larval requirements, and modern management practices allow to rear fish under conditions that guarantee the maximum growth rate, feed conversion efficiency, survival and, at the same time, the minimum problems related to infectious, nutritional and environmental diseases (Saraiva et al., 2015), poor growth of individuals, decrease in survival, malformations are often observed (Støttrup, 1993; Vadstein et al., 2013). Some of the problems associated with juvenile quality are visible only in later stages (Logue et al., 2000; Vadstein et al., 2013), so that it is important early recognize and take actions against factors that can damage production. For this reason, many studies are focusing on improving larval quality and its assessment. In this light, the H2020 PerformFISH (PFF) project aims, among many objectives, at optimizing and validating larval and juvenile quality indicators, specifically in gilthead sea bream - GSB (*Sparus aurata* L.) and European sea bass - ESB (*Dicentrarchus labrax* L.), respectively the third and fourth most farmed fish species in the EU by volume.

Fish histology is a tool largely exploited in fields such as basic biomedical research, ecotoxicology, environmental resource management and aquaculture (Wolf et al., 2015). Fish histological studies include a wide range of experimental topics, requiring the control and modulation of different rearing parameters (genetics of individuals, water parameters, diet) in order to evaluate their effects or assess the

Abbreviations: A_{org}, organ district architecture; CHI, Cumulative Histological Index; *d*, organ district descriptor; dph, days post hatching; ESB, European sea bass; GSB, Gilthead sea bream; MSSS, Multiparametric Semi-quantitative Scoring System; OCV, Organ condition value; PFF, PerformFISH; W_{org}, organ district weight.

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fish health status, as it is recognized that each biotic and abiotic factor has an impact on organ microanatomy (Saraiva et al., 2015). As these effects are often detectable with histology before the onset of manifest symptoms, therefore histological assessments can be effective tools for promptly recognize problems in larval batches and provide fish farmers with information about the impact of their rearing management.

Histological investigative methods are potentially exploitable within several experimental settings and paraphysiological adaptations. Pathological findings and changes should be compared (Sirri et al., 2018) and statistical analysis should be performed to interpret results. This is not possible by using the pure descriptive histology, therefore researchers tend to use other approaches such as the semi-quantitative scoring system and the quantitative method (histomorphometry). Although the quantitative system, carried out using image analysis software, offers numerous advantages that have been fully explained by Silva et al. (2015), the semi-quantitative scoring system is still a very widespread method for the assessment of histopathological changes in fish (Rašković and Poleksić, 2017). Obviously, this method requires a trained histopathologist which grades an alteration based on his consolidated experience. Typically, the range of the scores goes from a minimum of 0 or 1 (no alteration) to a maximum of 3, 5 or 10 (severe alteration) (Rašković and Poleksić, 2017). A semi-quantitative system can include single or multiple alteration, as well as it can consider one or more tissues/organs. In this regard several structured semi-quantitative scoring systems are currently used in fish studies (Poleksic and Mitrovic, 1994; Schwaiger et al., 1997; Bernet et al., 1999; Mitchell et al., 2012). The most cited is the system developed by Bernet et al. (1999): it is a standardized tool for the assessment of histological findings which can be applied to any organ. It was initially designed for the evaluation of pollution induced pathological changes in fish, but it is also exploited for other purposes such as the assessment of farmed fish health status (Saraiva et al., 2015, 2016). At the best of our knowledge, only one scoring system set up by O'Connell (1976) was specifically developed for the evaluation of fish larvae, and in particular for the diagnosis of starvation, but no other histological tools have been built with the purpose of evaluating the overall marine fish larval quality. This paper describes an original Multiparametric Semi-Quantitative Scoring System (MSSS) to estimate GSB and ESB quality in the hatcheries phase. This tool is developed so that it can be applied to different critical developmental stages throughout the hatchery phase, to check the condition of larval batches, helping fish farmers to understand the impact of their rearing management. The Cumulative Histological Index (CHI), calculated throughout the scores, returns a snapshot of a fish batch quality. The following paragraph will describe the structure of the scoring, the descriptors included and the principles that were followed to eliminate as much as possible the bias and to improve repeatability.

2. Design of the Multiparametric Semiquantitative Scoring System

The MSSS was developed within the PFF project activities (funded by Horizon2020), based on scientific literature data but especially on the authors' long experience in fish histology. It was aimed at assessing and comparing GSB and ESB batches of larvae and juveniles at different developmental stages (such as first feeding, flexion, end of the larval rearing, middle of the metamorphosis, and early juvenile), coming from different hatcheries, but it can be applied also for other marine fish species or modified also for fresh species.

Fish samples should be processed with standard histological techniques and stained with Haematoxylin and Eosin (HE) (other stains could be performed only to confirm some histological configuration). The specimens should be preferably placed on the midsagittal plane and observed under a light microscope (DMRB, Leica Microsystem, Germany) equipped with a digital camera (Nikon Fi3, Nikon Instruments Italia) and an imaging software NIS-Elements BR (Nikon Instruments Italia).

2.1. Scoring definition

An ordinal scoring method composed by whole numbers from 1 to 5 was chosen, in which 1 represents the normal condition and 5 the maximum extent of the evaluated phenomenon. It was decided to use a 5-levels range as less than this decreases sensitivity to detect group differences and more than this reduces repeatability (Gibson-Corley et al., 2013; Rašković and Poleksić, 2017). For some descriptors, the scoring criteria were defined quantitatively by means of an image analysis software, then, the measurements were divided into ranges corresponding to the scores from 1 to 5. Other descriptors were based on descriptive criteria, and the operator was properly trained to assign the correct score. Scoring progressive levels are shown in Table 1; moreover, the criteria for the definition of each descriptor score are detailed in the related paragraph to clearly discern each level and improve as much as possible the repeatability. For each different developmental stages, at least 20 subjects should be to evaluate by MSSS.

2.2. Organ districts and descriptors

The MSSS is composed by 18 descriptors belonging to 6 organ districts: gills, liver, anterior intestine, posterior intestine, pancreas and adipose tissue and excretory system (Table 1).

Most of the descriptors are organ-specific and have been selected because they are influenced by farming management practices (e.g., feed management, water quality etc.) and they are indicative of health status at larval stage. Only the inflammatory infiltrate, which may be detectable in any tissue, was considered as a descriptor in each organ district. In addition to descriptors, the organs general condition was considered: architecture of each organ districts has been assessed considering pathological progressive and regressive processes (nuclear alterations, necrosis, atrophy, deposits, hypertrophy, hyperplasia, etc., if they are not an integral part of the descriptor). In the following paragraphs, all the descriptors included in MSSS, their meaning and criteria for the score attribution will be discussed. Given the number of images taken, it was decided to accompany the description of the descriptors with photographic plates of GSB specimens only; the images have been chosen in order to guarantee an exhaustive comprehension of the relative descriptor, regardless of the age of the fish.

2.2.1. Gills (Fig. 1a, b)

2.2.1.1. Mucous cells (Fig. 1c). Mucous cells are large ovoid cells that are composed mostly of large apical mucous secretory granules. The mucous cells are commonly found in the filament epithelium, they are frequent in efferent edges and afferent edge, less in the interlamellar space and at the base of lamellae outer margin. In general, the number of mucous cells is higher in freshwater than seawater fish (Wilson and Laurent, 2002). Sarasquete et al. (2001) found that most mucous cells were PAS (Periodic Schiff Acid) and alcian blue (pH 2.5 and 0.5) positive in GSB larvae, indicating the presence of neutral and acid glycoconjugates (carboxylated and sulphated). In a minority of the mucous cells (the PAS-negative ones), bromophenol blue reaction (general proteins) was positive. Proteins rich in sulphydryl (-SH) and/or disulfide (-S-S-) groups related with the glycoprotein nature of the glycoconjugates present in mucous cells were also observed. No glycogen or lipids were detected. Also, in ESB histochemical studies showed that mucous cells in the primary filament epithelium contain acid, neutral and sulphated glycoconjugates (Diler and Çinar, 2009). Mucinous material elaborated by mucous cells in the epithelium of the gill arches and rakers of sea bass and sea bream is involved in lubrication, helping the smooth passage of food items through the pharynx, thus protecting the epithelium from mechanical injury (Grau et al., 1992; Murray et al., 1994; Tibbetts, 1997; Podkowa and Goniakowska-Witalinska, 2003). Mucous secretion in gills is also involved in fish immune responses

Table 1
Complete list of descriptors included in MSSS and score levels.

Descriptors	Scoring				
	1	2	3	4	5
GILLS					
Mucous cells (progressive changes)	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
Chloride cells (progressive changes))	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
Inflammatory infiltrate	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
LIVER					
Hepatocyte fat accumulation	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
Inflammatory infiltrate	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
ANTERIOR INTESTINE					
Desquamation	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
Thickness	*Normal*	*Slightly reduced*	*Reduced*	*Highly reduced*	*Extremely reduced*
Steatosis	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
Mucous cells	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
Inflammatory infiltrate	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
POSTERIOR INTESTINE					
Desquamation	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
Thickness	*Normal*	*Slightly reduced*	*Reduced*	*Highly reduced*	*Extremely reduced*
Supranuclear vacuoles	*Normal*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
Inflammatory infiltrate	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
PANCREAS and ADIPOSE TISSUE					
Visceral adipose tissue amount	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
Inflammatory infiltrate	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
EXCRETORY SYSTEM					
Calculi	*Absent*	*Small*	*Medium*	*Large*	*Very Large*
Inflammatory infiltrate	*Absent*	*Scarce*	*Moderate*	*Abundant*	*Highly abundant*
ARCHITECTURE	*Normal*	*Mild alterations*	*Moderate alterations*	*Severe alterations*	*Unrecognizable*

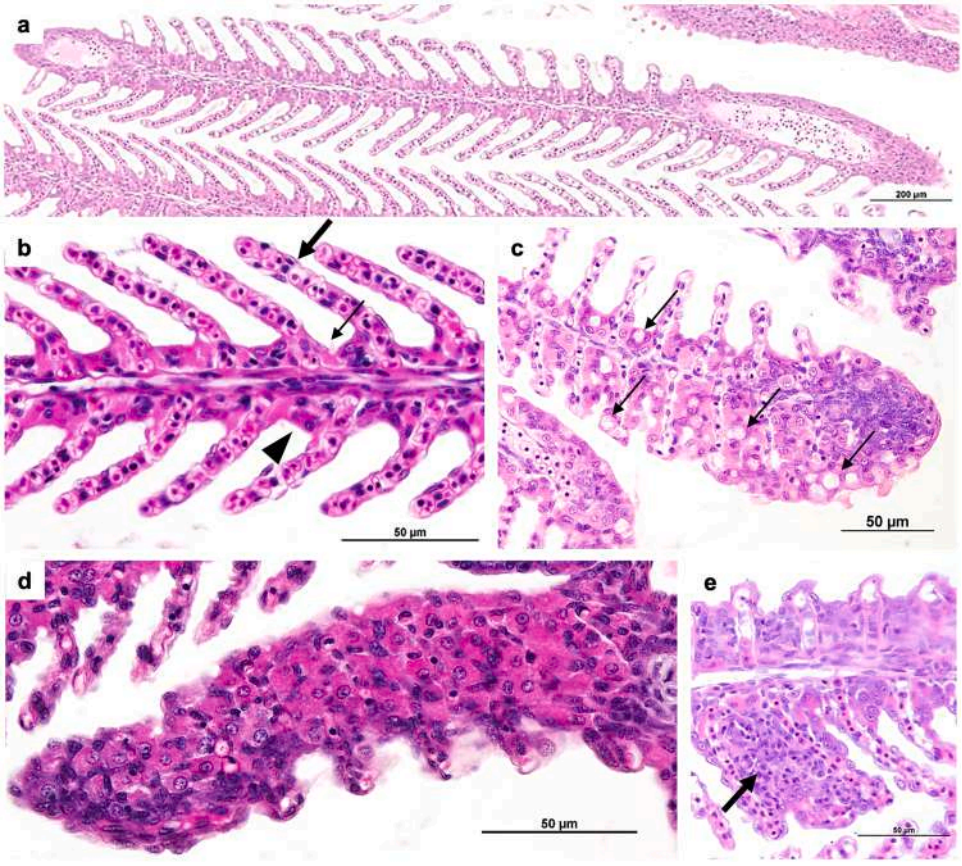

Fig. 1. GSB gills descriptors. a) 102 dph, normal primary gill filament, HE; b) 102 dph, secondary lamellae covered by epithelial cells (thin arrow), chloride cells (arrow heads) and mucous cells (thin arrow) are located within interlamellar sulci, HE; c) 85 dph, mucous cells (arrows) are well detectable and reactive, they are located also in the secondary lamellae; chloride cells hypertrophy and epithelial cell hyperplasia are also present, HE; d) 53 dph, severe hyperplasia and mild hypertrophy of chloride cells, HE; e) 92 dph, secondary lamellae fusion with inflammatory cell infiltration (arrow), HE.

(Alsafy, 2013); mucous cell hyperplasia in gills (also in skin) can be an indicator of pathological or inflammatory processes induced by pathogens (Dezfuli et al., 2007; Mahmoud et al., 2013), adverse environmental conditions and toxicant exposure (Rodrigues et al., 2019; Beegam et al., 2020) (Fig. 1).

The descriptor score is obtained by evaluating mucous cell hyperplasia in association to hypertrophy and metaplasia of epithelial cells:

1/5	absent- normal condition
2/5	scarce
3/5	moderate
4/5	abundant
5/5	highly abundant

2.2.1.2. Chloride cells (Fig. 1d). These are plump and slightly hyper-eosinophilic cells concentrated in the afferent region of the filament epithelium of the gill filament, within the lamellar sulci (Wilson and Laurent, 2002; Wolf et al., 2015). The term "chloride cell'' relates to their function in Cl^- elimination (Wilson and Laurent, 2002) but they are also known as "mitochondrion-rich cell" or "ionocyte" because they are involved not only in chloride secretion in seawater, but also in acid-base regulation and ammonia excretion (Hiroi and McCormick, 2012). Changes of these cells occur in response to irritating factors exposure, especially when chronic. The effects of heavy metals, ammonia intoxication, excessively high or low water pH, and parasitic infestations are thought to be the cause of excessive proliferation (Strzyzewska et al., 2016). For the purpose of this study chloride cells were considered hyperplastic when proliferating along the lengths of lamellae in a basal to apical direction as indicated in Wolf et al., 2015, and hypertrophic when larger than the mean size of a normal chloride cell (about 8 μm in the analyzed samples). Score criteria are shown below:

1/5	absent	normal condition
2/5	scarce	hypertrophic chloride cells but not yet proliferated along the secondary lamellae
3/5	moderate	the chloride cells were hypertrophic with a beginning of proliferation along the secondary lamellae
4/5	abundant	the chloride cells are hypertrophic with marked proliferation along the secondary lamellae
5/5	highly abundant	hypertrophic chloride cells with severe proliferation along the secondary lamellae and alteration of the structure

2.2.1.3. Inflammatory infiltrate (Fig. 1e). In many fish species, including GSB and ESB, the basilar areas of gill filaments, gill arches, and pharyngeal mucosae contain dense populations of resident lymphocytes, eosinophilic granular cells and acidophilic granulocytes (Gills Associated Lymphoid Tissue – GIALT) so it is tempting to diagnose such constituent infiltrates as gill inflammation (branchitis). However, according to Wolf et al. (2015) actual branchitis tends to involve the distal two-third of the filaments in addition to the more proximal regions and other features of inflammation (e.g., necrosis of pavement or endothelial cells, vascular congestion) often accompany the leukocytic infiltrates. Leukocyte infiltration could be considered as inflammatory responses that occur in fish exposed to a severe type of stress (Rosety-Rodríguez et al., 2002). The presence of toxicant can cause this reaction in fish gills; as example, a 28days-exposure to Polycyclic aromatic hydrocarbons induces infiltration of inflammatory cells albeit in relatively small and sparse foci in juvenile ESB (Martins et al., 2016). Score attribution criteria for the assessment of inflammatory infiltrate are shown below:

		N. of inflammatory cells (mean number of inflammatory cells counted in 4 fields, at 400 × magnification)
1/5	absent	from 0 to 5
2/5	scarce	from 6 to 20
3/5	moderate	from 21to 50
4/5	abundant	from 51 to 150
5/5	highly abundant	more than 150

2.2.2. Liver (Fig. 2a)

2.2.2.1. Hepatocyte fat accumulation (Fig. 2b). Liver is the most important regulator of lipid metabolism, including both the synthesis and degradation of fatty acids, thus imbalances in the dietary fatty acids could modify the function and morphology of this organ. In certain fish species, including gilthead seabream and European seabass, liver also functions as a main energy reservoir, frequently in the form of triacylglycerols (TGs) (Kaushik, 1997 quoted by Caballero et al., 2004). Also in fish larvae, as in adult fish, the liver plays this role. Mature hepatocytes have been observed in the liver of several species from first feeding (Tanaka, 1969; Guyot et al., 1998; Micale et al., 2008; Rønnestad et al., 2013). When dietary lipid/energy exceed the physiological capacity of the hepatic cells to oxidize fatty acids, the result is a large synthesis and accumulation of TG in cytoplasmic vacuoles, potentially leading to steatosis. (Caballero et al., 2004). This abnormal fat retention (cellular pathology) involves a series of pathological alterations up to the cell necrosis, microscopically demonstrated by the large volume of hepatocytes (globose appearance), the cytoplasm entirely filled by lipids, and the nucleus moved to the margins of the cell body (close to the plasma membrane). In GSB, the diet lipid content, the replacement of fish meal/oil with alternative vegetable sources and, most of all, the zootechnical forcing could lead to significant liver changes. Anyway, since the point at which hepatic lipid accumulation becomes deleterious to fish is currently indeterminate and probably quite variable within and among fish species, the term steatosis/lipidosis should be used only when it is possible to demonstrate cellular alterations induced by an excessive accumulation of lipids, such as cell membrane rupture or saponification (Wolf et al., 2015). In this study, the score setting of was initially defined by using the image analysis software NIS-Elements BR (Nikon instruments Italia) and calculating the white percentage present in the binary image as indicated below. The quantitative analyses should only help the initial evaluator training (Fig. 2).

		White percentage (mean percentage of 4 fields at 400 × magnification
1/5	absent	0–10 %
2/5	scarce	11–25 %
3/5	moderate	26–40 %
4/5	abundant	41–55 %
5/5	highly abundant	> 55 %

2.2.2.2. Inflammatory infiltrate (Fig. 2c). Inflammatory processes in liver have been described in fish exposed to polychlorinated biphenyls, polycyclic aromatic hydrocarbons, pesticide polluted water, pulp and paper mill effluents, treated sewage, dispersed oils (Peters et al., 1987; Bucher and Hofer, 1993; Adams et al., 1996; Schwaiger et al., 1997; Bernet et al., 2004; Agamy, 2012; Fu et al., 2017). Liver infiltration of

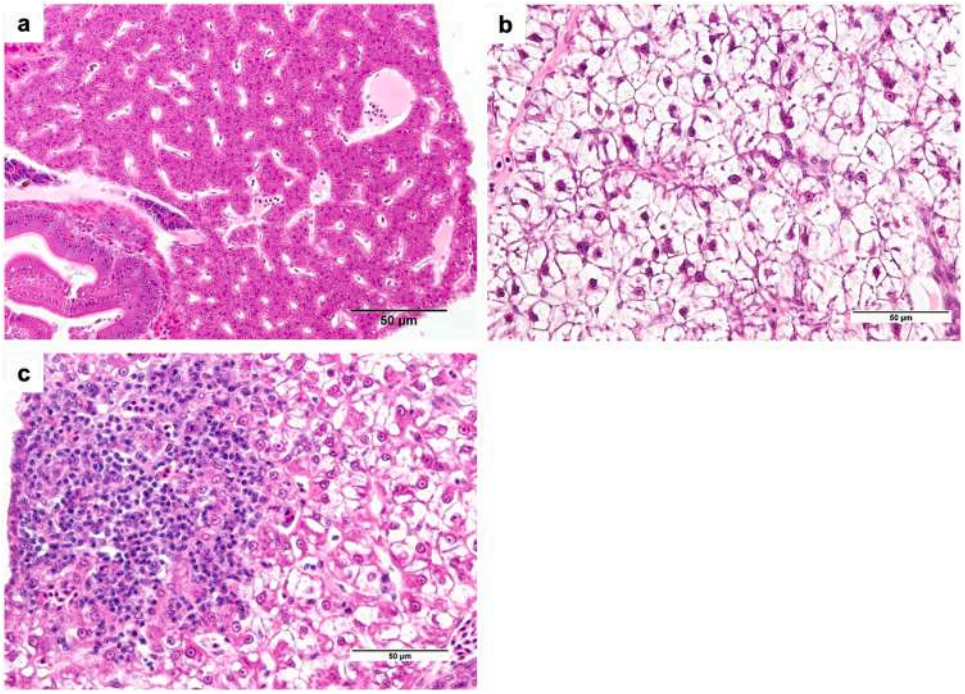

Fig. 2. GSB liver descriptors. a) 92 dph, normal liver, hepatocytes have polygonal shape and central nuclei, cordon structure is evident, HE; b) 87 dph, fat accumulation in hepatocytes, the size is increased, and most of the nuclei are located at the cell periphery, HE; c) 100 dph, large focal lymphocytic liver infiltrate, HE.

seabream acidophilic granulocytes has been detected in GSB exposed to waterborne cadmium (Guardiola et al., 2013). Nutritional factors can also trigger inflammatory reactions; Rios et al. (2007) found some lymphocyte infiltration near karyopycnotic or karyolytic hepatocytes after 180 days of food deprivation in Neotropical trahíra (*Hoplias malabaricus)*. In a study conducted by Rašković et al. (2016), the presence of some inflammatory cells, such as EGC and leukocytes, inside the liver parenchyma resulted higher in common carp (*Cyprinus carpio* L.) fed with extruded feed than those in common carp fed with fed with grain cereals. Scores are attributed as indicated in Section 2.2.1.

2.2.3. *Anterior intestine (Fig. 3a)*

2.2.3.1. Desquamation (Fig. 3b). At hatching, the epithelium of the alimentary canal of GSB and ESB larvae is made up of a mono-stratified layer of columnar cells. The anterior intestine becomes distinguishable from the other two portions (foregut and posterior intestine - hindgut) at about 3–4 days post hatching (dph). At this age the anterior intestine is lined with an epithelium consisting of columnar cells showing the brush border (Sarasquete et al., 1995; Rekecki et al., 2009). As the exogenous feeding start and with the progression of development, the epithelium thickens at several points, resulting in an undulating surface. The underlying connective tissue enter the thickened points of the epithelium to form true mucosa folds, which progressively grow in size and ramifications (García Hernández et al., 2001) The anterior intestine is the portion active in lipid absorption in fish larvae (Sarasquete et al., 1993; Elbal et al., 2004; Rekecki et al., 2009) but, in addition to digestion and nutrient absorption, in general intestinal functions in fish includes hormone secretion, immune protection and water and salt transfers for hydro mineral homeostasis (Giffard-Mena et al., 2006). Desquamation in fish intestine can be caused by different factors; as example, high lipid accumulation in enterocytes can lead to the desquamation and degeneration of the epithelium both in larvae (Padrós et al., 1993; Crespo et al., 2001) and adult fish (Burrells et al., 1999; Olsen et al., 2000). Moreover, the intestine is a very important absorption site for the toxic compounds; the exposure to certain concentrations of heavy metals (lead, cadmium, zinc, copper) leads to the desquamation of living epithelial cells (Polistovskaya et al., 2018). Pathogens can also provoke intestinal epithelium desquamation (Dezfuli et al., 2016; Sitjà-Bobadilla et al., 2016). Desquamation leads to a decrease in the total surface of absorption and absorption capacity, which in turn leads to a disorder of intestinal absorption processes (Polistovskaya et al., 2018). Score attribution criteria for the assessment of inflammatory infiltrate are shown below (Fig. 3):

1/5	absent	normal condition, no cell detaching from the epithelium was observed
2/5	scarce	few cells in the detachment, no cells detached within the lumen, the process is not diffuse
3/5	moderate	cells both in detachment phase and within the lumen, affected area is reduced or medium
4/5	abundant	abundant cells are found inside the lumen, the alteration involves at least 50% of the epithelium
5/5	highly abundant	cells desquamation and their presence in the lumen is very abundant, the alteration is very diffuse more than the 50% of the epithelium

2.2.3.2. Thickness (Fig. 3c). In addition to infectious etiology, the variation in intestinal epithelium height may be due to the larval nutritional conditions. Studies were out to elucidate the effect of starving and delayed first feeding in larvae of several fish species (Bisbal and Bengston, 1995; Theilacker and Porter, 1995; Green and McCormick, 1999; Gisbert et al., 2004; Ostaszewska et al., 2006; Chen et al., 2007) and results suggested that the condition and height of intestinal epithelial cells are good indicators for malnutrition or starvation, as enterocyte height decreases with food deprivation, and proteolysis of the enterocytes eventually leads to low absorption of nutrients due to reduced surface area (Green and McCormick, 1999). Diet components also affect intestinal thickness: Rekecki et al. (2009) observed that

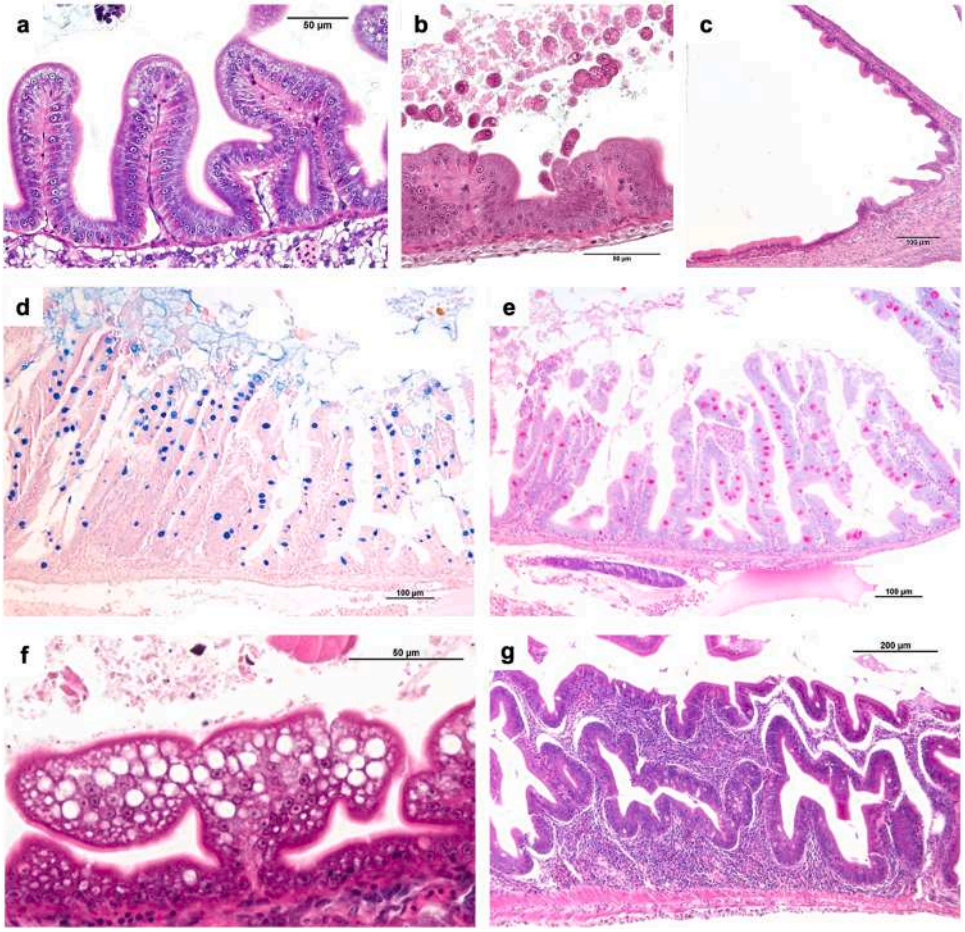

Fig. 3. GSB anterior Intestine descriptors. a) 38 dph, normal anterior intestine, folds are developed, enterocytes are columnar and equipped with microvilli, HE; b) 40 dph, severe presence of desquamated cells in the intestinal lumen, some cells are in the detachment phase, HE; c) 50 dph, thinning of the intestinal mucosa, the folds are reduced, and the height of the enterocytes decreased becoming cubic in some portion, HE; d) 95 dph, Alcian Blue positive mucous cells in anterior intestine and e) PAS positive mucous cells; f) 38 dph, steatosis of the anterior intestine, small and large lipid droplets in enterocytes, HE; g) 98 dph, severe inflammatory infiltration mainly lymphocytic in lamina propria and epithelium, HE.

control larvae exhibited a slightly higher intestinal epithelium in the anterior intestine compared larvae fed with a germ-free diet. The midgut of control larvae consisted of a cuboidal to columnar epithelium, whereas the midgut of both groups of germ-free larvae was lined with a cuboidal to squamous epithelium. This descriptor was detailed below:

1/5	normal	normal condition, the intestinal folds were well developed (also based on the developmental stage of the fish), the epithelial cells were columnar
2/5	slightly reduced	reduction in the height of the folds was observed, but with normal enterocyte height
3/5	reduced	the folds were completely flattened, but the height/volume of the enterocytes was normal
4/5	highly reduced	the folds were completely flattened, enterocytes were from slightly lowered to almost cuboid
5/5	extremely reduced	the folds were completely flattened, the enterocytes had a cuboid to squamous shape

2.2.3.3. Steatosis (Fig. 3f). The anterior intestine is the principal region involved on the absorption of lipid (Sarasquete et al., 1995; Elbal et al., 2004). According to Deplano et al. (1989), in the enterocyte, the absorbed lipids appear in 2 granule forms (small, about 30–100 nm, found in wild fish and 50–400 nm in cage-reared fish) and droplets. The presence of small lipid granules in enterocytes are probably transportation forms as very low-density lipoproteins (VDLD) or chylomicrons (Deplano et al., 1989; Sarasquete et al., 1995). In sea bream and sea bass larvae, it has been found that enterocytes absorb lipids from the diet from the first feeding, but since these cells have a low capacity for lipoprotein synthesis, the lipids are accumulated in the form of larger free lipid droplets. The rapid development of the enterocytes during larval growth is combined with increasingly effective lipoprotein synthesis, accompanied by a considerable decrease in number of large lipid vacuoles in the enterocytes (Deplano et al., 1991; Sarasquete et al., 1995). Thus, the accumulation of larger lipid droplets in the enterocytes may be considered a temporal storage of lipids caused by an inability to mobilize them. It may be due to insufficient lipoprotein synthesis or insufficient synthesis of phospholipids required for lipoprotein synthesis (Kjørsvik et al., 1991; Sarasquete et al., 1995; Olsen et al., 2000; Wassef et al., 2016). Studies concluded that lipids droplets are absent in starving larvae (Rekecki et al., 2009). Deplano et al. (1989) found that small lipid granules are common while larger lipid droplets are rare in anterior intestine enterocyte in wild sea bass; the latter gave the appearance of clear homogeneous pools, located in the supranuclear region or at the very base of the cell. In the same study an overload of lipids was seen in the epithelium; large lipid droplets, similar to those found in wild fish but larger, were observed. The author concluded that the primary cause of this phenomenon was the inadequacy of the artificial food used in fish-farming, and in particular an unbalance in the lipid fraction. Intestinal steatosis does not appear to be a cause for alarm, in view of the low mortality associated with it, but a deteriorated intestinal mucosa no longer plays an effective role as a selective barrier. Certain molecules can penetrate the internal medium and provoke indirect disturbances. Moreover, the decrease in absorptive capacity could lead to feed wastage.

Score criteria for this descriptor are reported here below:

1/5	absent	normal condition, absence of lipid droplets
2/5	scarce	the droplets were small and not diffuse, clearly visible but did not alter the cell architecture
3/5	moderate	droplets were diffuse, small, and numerous to slightly alter the cell structure or few but large, slightly altering the structure
4/5	abundant	the droplets were very diffuse and large, considerably altering the enterocytes architecture but without causing them to rupture
5/5	highly abundant	the droplets were very diffuse and large, they sometimes caused the enterocyte rupture or mucosa alteration

2.2.3.4. Mucous cells (Fig. 3d, e). Mucins play an important role in protecting the mucosa against pathogens attack and physical and chemical damage, moreover in the digestive tract, mucus also acts as enzymatic support, and not only as a lubricant. The time of appearance of mucous or goblet cells in the teleost digestive tract varies among species. In gilthead seabream, digestive regions are histologically differentiated from 4 dph (Sarasquete et al., 1995) and goblet cells appear in the anterior intestine from the exotrophic phase; some of them contain neutral mucins and others acid (sialomucin-type and sulphated) or mixed mucin types (Sarasquete et al., 1995; Elbal et al., 2004;). Also, in European seabass intestinal goblet cells are first found in the intestine during the exotrophic phase and are rich in neutral (García Hernández et al., 2001) or acid muco-substances (García Hernández et al., 2001). According to Sarasquete et al. (1995), inter- and intraspecific differences in the content of muco-substances in the digestive goblet cells could be related to different feeding habits. The number of mucous cells in the digestive tract normally increases with larval development, moreover the number of these cells varies due to several factors as the diet (Baeza-Ariño et al., 2016; Torrecillas et al., 2017), microplastic and toxicant exposure (Pedà et al., 2016), presence of pathogens (Redondo and Álvarez-Pellitero, 2010; Xu et al., 2019). The scoring criteria are described here below:

		N. of mucous cells (mean of 4 fields, 400 × magnification)
1/5	absent	from 0 to 5
2/5	scarce	from 6 to 20
3/5	moderate	from 21to 40
4/5	abundant	from 41 to 60
5/5	highly abundant	more than 60

2.2.3.5. Inflammatory infiltrate (Fig. 3g). Lymphocytes, plasma cells, granulocytes and macrophages are present in all parts of the teleost digestive system, most extensively in the intestine (Gut-Associated Lymphoid Tissue - GALT) in and under the epithelium aiming at protecting from infection and prevent penetration by pathogens. At the same time, over-reaction against food antigens and normal microbial flora may influence the recruitment of these cells (Abelli et al., 1997). According to Wolf et al. (2015), it is possible that some types of histopathologic changes are underreported because they have insufficient precedence in the literature; that is, investigators tend to search for types of lesions that have been described commonly in the past, whereas less frequently reported changes are not necessarily at the forefront of their awareness. Intestinal inflammation (focal or diffuse expansion of the submucosa by leukocytic infiltrates) is included in this category. Enteritis characterized by a widening of the lamina propria infiltrated by a mixed population of inflammatory cells identified as lymphocytes, macrophages, eosinophilic and granular cells, are reported in teleosts as Atlantic salmon, rainbow trout and common carp fed a diet based on partial replacement of fish meal by soybean, attributing this alteration to soya saponins (Uran et al., 2008, 2009; Øverland et al., 2009). Similarly, several authors in GSB (Sitjà-Bobadilla et al., 2005; Bonaldo et al., 2008; Kokou et al., 2015) have also found dilation of the submucosa with some infiltration of eosinophils. In contrast, other species, such as Atlantic halibut, channel catfish, Egyptian sole and turbot (Grisdale-Helland et al., 2002; Evans et al., 2005; Bonaldo et al., 2006, 2011) did not exhibit any inflammatory response in the intestinal mucosa consequently to the inclusion of soybean meal in the diet. In addition, ESB seems to be less sensitive to certain soy- anti-nutritional factors, which induce intestinal disturbances in salmonids (Tibaldi et al., 2006; Bonaldo et al., 2008; Bonvini et al., 2018). Scores are attributed as indicated in Section 2.2.1.

2.2.4. Posterior intestine (Fig. 4a)

2.2.4.1. Desquamation (Fig. 4b). As regards the present parameter we can refer to the description reported for Desquamation in Anterior Intestine (Section 2.2.3) (Fig. 4).

2.2.4.2. Thickness (Fig. 4c). The posterior intestine (hindgut) is lined by a cuboid-columnar epithelium similar to that of the anterior intestine, with a tall brush border on the apical surface (Sarasquete et al., 2001; Rekecki et al., 2009). At the first feeding, the epithelium shows points of thickening. These progressively become mucosa folds with an axis of loose connective tissue (García Hernández et al., 2001). A thin intestinal epithelium lacking supranuclear protein inclusions in the posterior intestine is symptom associated with poor assimilation (Yúfera et al., 1993, 2000; Sarasquete et al., 1995). In the study of Rekecki et al. (2009), differently from the anterior intestine, the posterior of the control larvae was characterized by a cuboidal to columnar epithelium with a visible brush border, whereas in germ-free static larvae it was lined by simple columnar epithelium with a very tall brush border. The posterior intestine of rotating germ-free larvae was lined by a simple squamous to cuboidal epithelium. The score attribution criteria for this descriptor are like those of anterior intestine Thickness (Section 2.2.3).

2.2.4.3. Supranuclear vacuoles (Fig. 4d). During the transition from endogenous to exogenous feeding, the posterior intestine has a basic nutritional role in absorbing protein macromolecules by pinocytosis as an alternative pathway until the development of the stomach and acid digestion takes place. Thus, the presence of supranuclear vacuoles with acidophilic inclusions is a typical feature of the posterior intestine of fish larvae during early life stages (Yúfera et al., 1993; Sarasquete et al., 1995; Gisbert et al., 2008). These inclusions are strongly positive with PAS stain and slightly with Alcian blue (pH 2.5) indicating the presence of neutral mucopolysaccharides (and/or glycoproteins) and carboxylated acid mucopolysaccharides. They are also positive to bromophenol blue, confirming the presence of proteins (Yúfera et al., 1993; Sarasquete et al., 1995). Although supranuclear vacuoles are normal, their overdevelopment is described in larval sea bass as a pathological condition by Deplano et al. (1999), potentially resulting in cytological deterioration combined with cell degeneration and pronounced epithelial abrasion. According to them, the hypertrophy of supranuclear vacuoles may result from an excess of undigested proteins in the posterior intestine caused by an excessive proportion of proteins in the feed ration, partial or total indigestibility of some proteins by gastric or pancreatic enzymes or a qualitative or quantitative deficit of intraluminal proteolytic agents. Preliminary histochemical studies on GSB supranuclear vacuoles carried out by authors, showed that, in some cases, the content of these vacuoles appears as a brownish lipofuscins-like pigment when observed in white sections; moreover, they result positive to PAS stain, Lillie's Nile blue, and Schmorl's stain, confirming the presence of lipofuscins. Although in most of the studied species the number and size of supranuclear vacuoles decreased as the

stomach differentiated and extracellular digestion take place (Gisbert et al., 2008), they have been described in epithelial cells of the sea bass rectum during phase IV (55–61 dph) (García Hernández et al., 2001) as well as in the rectum of larvae and adults of other teleosts including *S. aurata* (Elbal and Agulleiro, 1986; Cataldi et al., 1987; Sarasquete et al., 1995). Details of this descriptor are reported here below:

1/5	normal	normal condition, without visible supranuclear vacuoles
2/5	moderate	vacuoles occupy the entire luminal portion of the cell without altering its structure, the content is more abundant, acidophilic, not compact
3/5	abundant	the vacuoles size was slightly increased, widening the apex of the enterocyte, the contents are strongly acidophilic, compact
4/5	very abundant	the vacuoles are medium or large, enlarging the supranuclear portion of the enterocyte, the content is compact, strongly acidophilic up to orange, rarely brownish
5/5	highly abundant	very large vacuoles (also reaching 15–20 μm), dilating the supranuclear portion of the cell, sometimes there is a reduction of the intestinal lumen, the content is dense, orange/brownish also without staining

2.2.4.4. Inflammatory infiltrate (Fig. 4e). As regards the present parameter we can refer to the description reported for Inflammatory infiltrate in Anterior Intestine (Section 2.2.3).

2.2.5. Pancreas and visceral adipose tissue (Fig. 5a)

2.2.5.1. Visceral adipose tissue amount (Fig. 5c). In mammals, obesity is associated with a number of health problems that are often summarized together as metabolic syndrome and involves the development of insulin resistance, type 2 diabetes, cardiovascular disease, fatty liver disease and systemic inflammation (Galic et al., 2010). In ESB and GSB the replenishment of body fat stores is seasonal, but current aquaculture practices largely increase fat deposition in fat storage tissues, leading to production and indirect selection of specimens with fatty characteristics which may compromise both their welfare and their final product quality (Cruz-Garcia et al., 2009; Salmerón, 2018). Lipids accumulate in teleost mainly in the form of triglycerides, in different anatomical sites (visceral organs, liver, subcutaneous tissues, red and white muscles, brain, pancreas, esophagus, mandible, cranium and tail fin) depending on the species, the nutritional state, the life-stage and the physiological status, but the preferential lipid storage sites are the perivisceral fat, subcutaneous fat, muscle and liver (Flynn et al., 2009; He et al., 2015; Weil et al., 2013, Salmerón, 2018). Although this topic is currently

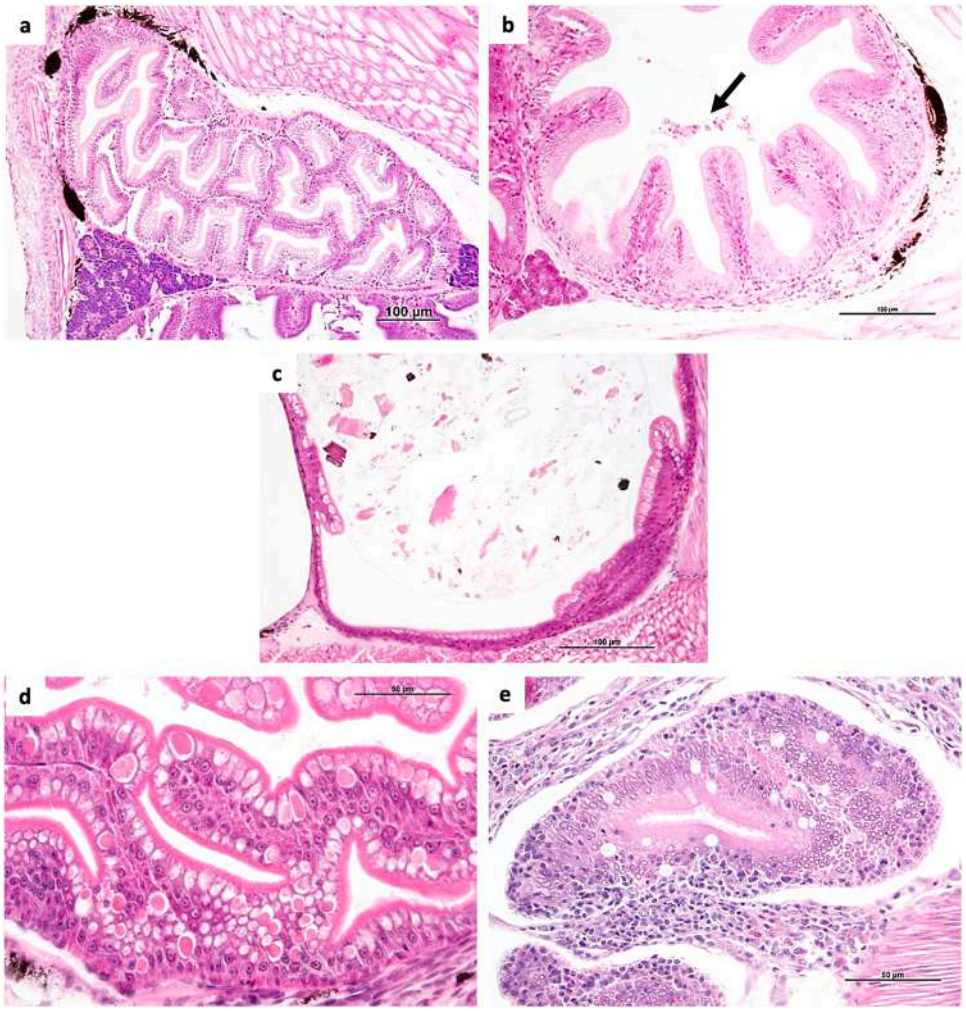

Fig. 4. GSB posterior Intestine descriptors. a), 35 dph, normal posterior intestine. Folds are developed, enterocytes are columnar and equipped with microvilli, small vacuoles with light content are present, HE; b) 40 dph, slight presence of desquamated cells (arrow) in the intestinal lumen, HE; c) 35 dph, thinning of the intestinal mucosa, the folds are almost disappeared, and the height of the enterocytes decreased becoming cubic in some portion, small supranuclear vacuoles are still present, HE; d) 29 dph, presence of medium/large supranuclear vacuoles, with strongly acidophilic dense content, HE; e) 95 dph, abundant inflammatory infiltration mainly lymphocytic in lamina propria, HE.

attracting researchers' attention, the pathophysiological role of the adipose tissue accumulation in fish still needs to be explored from some point of view. However, different authors found that some common obesity pathophysiological pathways are shared in fish and mammals (Oka et al., 2010; Ichimura et al., 2013). In fish, some nutrients that are currently used for fish feed, such as vegetable oils, induce more hypertrophic adipose tissue growth, lipolysis and lipid accumulation than do fish oils, provoking an excess of fat deposition that decreases the product quality and could lead to a lower insulin sensitivity; visceral far depots are particularly influenced by this replacement in juvenile GSBs (Cruz-Garcia et al., 2011; Todorčevic et al., 2008). The score descriptor criteria are shown below (Fig. 5):

1/5	absent	no adipocytes were detected in visceral cavity
2/5	scarce	few and homogenous in size adipocytes were found mostly scattered or in small aggregates, interspersed with the pancreatic parenchyma, mostly in the posterior portion and in abdominal position with respect to the intestine
3/5	moderate	adipocyte size was still homogenous and the cells were organized in aggregates involving the posterior portion of the pancreas
4/5	abundant	the size of the adipocytes is heterogeneous, adipose tissue was diffused in pancreatic parenchyma and visceral cavity but without invade the cranial part
5/5	highly abundant	the size of the adipocytes is heterogeneous, and the adipose tissue was diffused in pancreatic parenchyma and visceral cavity, invading the cranial part

2.2.5.2. Inflammatory infiltrate (Fig. 5b). Although few information has been found in literature concerning this descriptor, it has been included in the MSSS as previous author observations shown that apparently healthy GSB post-larvae have a significantly higher number of pancreatic inflammatory cells than that recorded in those reared in the same area 20 years ago (Beraldo and Galeotti, 2013; Beraldo et al., 2018). This could be the result of a changed nutritional scenario, both in terms of diet composition due to the replacement of animal origin ingredients with others of vegetable origin, and in the management of feeding, characterized by zootechnical forcing in order to promote rapid growth (Beraldo et al., 2018). Score attribution is described in Section 2.2.1.

2.2.6. Excretory system (Fig. 6a–c)

2.2.6.1. Calculi (Fig. 6d). Nephrocalcinosis is a chronic condition affecting the kidney, characterized by the presence of mineral deposits within the renal tissue. Farmed fish are more sensitive to environmental factors as they live in a restricted environment, and they are also often subjected to other contingent stressors. Nephrocalcinosis is usually associated with increased levels of free CO_2 in the water and coexisting conditions, such as decreased levels of dissolved oxygen and reduced pH (Vatsos and Angelidis, 2017). It has been suggested that a mechanism leading to these lesions in the kidney could be the precipitation of the calcium hydrogen phosphate present in the urine when the urinary pH increases (Roberts and Rodger, 2001). This change could occur in fish exposed to elevated CO_2 concentration. However, several studies showed that other mechanisms are involved in the onset of the nephrocalcinosis, such as the high levels of calcium coupled with phosphorous and magnesium in the diet (Richardson et al., 1985 quoted by Vatsos and Angelidis, 2017). Within the nephrocalcinosis condition, urinary calculi, mainly calcium phosphate crystals, Ca5(PO4), can be observed in the urethra or urinary bladder. According to Moretti et al. (1999) calculi are the earliest signal of poor quality in a larval batch, and they are correlated with environment-induced stress. When a large calculosis (involving more than 40 % of the fish of a batch), is observed, it can be interpreted as a sign of poor rearing conditions which typically will result in a low survival rate and environmental and unsuitable feeding parameters (excessive water currents due to wrong aeration or water inflow, disproportionate prey size, insufficient light intensity, dangerous levels of some water quality parameters), or even, when the total observation overcome 30% during the first feeding days (5–15) is recommended to empty the tank and restart the culture with a new population. In the MSSS calculi are considered as a descriptor related to the excretory system condition. The score attribution is based on their dimension as indicated here below (Fig. 6):

	Calculi
1/5	Absent
2/5	Small, from traces to 50 μm
3/5	Medium, from 51 μm to 200 μm
4/5	Large, from 201 μm to 400 μm
5/5	Very large, larger than 400 μm

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(continued)

2.2.6.2. Inflammatory infiltrate (Fig. 6e). Due to of the high degree of resident cellularity in the normal renal interstitium, the identification and characterization of renal inflammatory infiltrate can be challenging. The most easily appreciated form of interstitial inflammation is the granulomatous type (Wolf et al., 2015). However, in this study the detection of granulomas was included in the evaluation of organ architecture. The inflammatory infiltrate, where clearly distinguishable, was attributed shown in Section 2.2.1.

3. Cumulative histological index and organ condition value calculation

A single value giving general information about the quality of a fish batch at a precise time, named Cumulative Histological Index (CHI), is obtained applying a weighted sum summarizing all the MSSS descriptors. First, for each organ, a value resulting by the product of the mean of organ district descriptors ($\bar{d}$; range 1–5) and the organ architecture (*A;* range 1–5) is calculated (Formula 1). This value is named Organ Condition Value (OCV) and identifies the degree of histological alteration of an organ district. OCV can range from 1 to 25, where 1 is the best situation and 25 the worst condition of the organ. For the CHI calculation (Formula 2), the OCVs of each organ district are weighted on weights attributed to the organs (*W*) and summed; CHI value varies from 1 to 25 too.

Formula 1. OCV calculation

$$OCV = \bar{d}_{org} \times A_{org}$$

Formula 2. CHI calculation

$$CHI = \sum OCV_{org} \times W_{org}$$

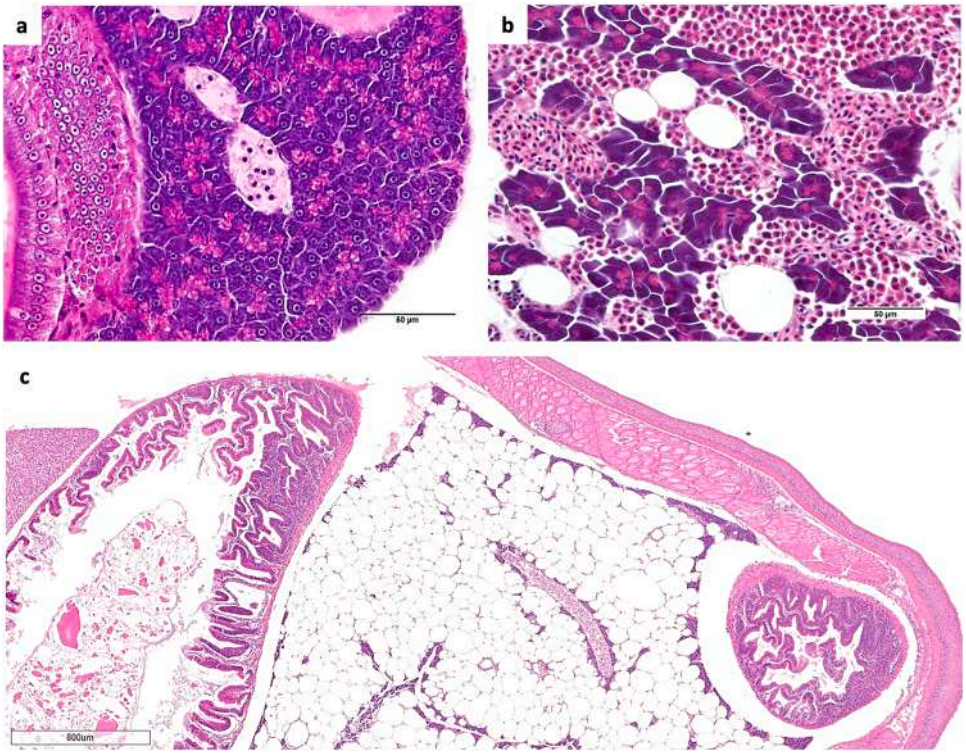

Fig. 5. GSB pancreas and adipose tissue descriptors. a) 80 dph, normal pancreas, acinar cells with dark basophilic cytoplasm and bright, eosinophilic, secretory zymogen granules, HE; b) 90 dph; inflammatory cells, predominantly acidophilic granulocytes, infiltrating the pancreatic parenchyma, HE; c) 98 dph, visceral cavity, the adipose tissue invades most of the cavity and pancreas, the size of the adipocytes is not uniform, HE.

A_{org} – organ district architecture; CHI – Cumulative Histological Index; $\overline{d}_{org}$ – mean of organ district descriptors; OCV – Organ Condition Value; OCV_{org} – Organ Condition Value of all organ districts; W_{org} – organ district weight .

The organ weight is a whole number ranging from 1 to 3, considering the vital importance of that organ and the related expert knowledge of the authors, as reported here below:

Organ district	Weight
GILLS	3/11
LIVER	1/11
ANTERIOR INTESTINE	1/11
POSTERIOR INTESTINE	1/11
PANCREAS	2/11
EXCRETORY SISTEM	3/11

Each weight is divided by the sum of all weights so that their sum is equal to 1. The CHI can be used in its entirety or partially, according to the developmental stage of the fish (see Section 4.1) and the research purposes: when complete MSSS is applied, the weight value is divided by 11. A CHI ranging between 1 and 3.5 is indicative of fish healthiness, while higher CHI point out the presence of pathological alterations, which may or may not be biocompatible, in relation to the affected body district, as deduced from the different organ weight in the CHI formula. The maximum value of 25 is only virtually achievable, as the severity of the lesions realistically cannot be biocompatible close to this value. The threshold of 3.5 was set on the basis of scenario simulations (based on different virtual score combinations) and, in addition, it should be considered as a warning light for the fish farmer to early adopt management strategies in fish batch before the situation becomes very serious or irreversible.

4. Indications and considerations for the use of the msss

4.1. Developmental stages

The MSSS is built to be applicable to different stages of the hatchery phase; anyway, the different degree of ontogenetic development, does not allow to evaluate all these stages in the same way, the adaptations that must be applied to the MSSS are indicated below:

- the organ district “Gills” is not included in MSSS when used on first feeding and flexion stages as this organ is structurally poorly differentiated into primary and secondary gill filaments; this has to be considered in CHI calculation, in particular in the sum of the weights, in fact, the weight of the gills is 3, so each weight has to be divided for 8 in these stages, not for 11;
- the descriptor “Inflammatory Infiltrate” (in all the organ districts) is not included in MSSS when used on first feeding and flexion stages;
- the complete MSSS can be applied to end of larval rearing, middle of the metamorphosis and juvenile stages.

4.2. Sampling and sample preparation

The quality of the tissue is essential for the success of the evaluation. Although a trained operator can, to some extent, recognize and compensate irregularities in histological preparations, poorly fixed and preserved samples undergo post-mortal lysis that partially or totally compromise the analysis, or lead to artifacts that make complex the interpretation of the findings (Fig. 7a–d). In order to collect good quality specimens from hatcheries and standardize procedures, a Sampling Manual was prepared within the PFF project. Briefly, in hatchery, fish should be caught with appropriate nets, sacrificed with an overdose of tricaine methane sulfonate MS-222 (50–100 mg/L) and immediately transferred in Bouin’s solution (Bio-Optica, Italy) in 1:10 ratio. Fixation time depends on size of fish and ranged from 1 h (first feeding larvae) to

24 h (juveniles). After fixation, samples should be preserved in EtOH 70 % and quickly processed. Based on author's lab experience, the Bouin's solution allows an excellent quality of histological specimen due to a mild decalcifying of acetic acid, especially for first feeding and flexion subjects (PerformFISH – H2020, 2017).

4.3. Sample evaluation and statistical analysis

As the semi-quantitative scoring depends on the observer training, it could potentially cause some evaluation bias which may influence the data and conclusions obtained, and so some precautions must be taken in order to constrain these biases, as suggested by several authors (Gibson-Corley et al., 2013; Meyerholz and Beck, 2018). The risk of having judgment subliminally influenced by information cues from the study, belongs to experts of all levels; the blind observation (masking) of the sample reduces this risk (Gibson-Corley et al., 2013; Meyerholz and Beck, 2018). The "diagnostic drift" is a situation in which the assignment of scores may vary slightly in consistency through the scoring process. This can happen in several situations such as cases where there is a large number of samples (Gibson-Corley et al., 2013). In applying the MSSS, in consideration of the fact that for the purposes of the PFF project was required the analysis of many fish batch, it was decided to analyze 20 fish per each developmental stage/batch. The choice of this sample size was a compromise between the need to evaluate an adequate number of fish from a huge larval batch but at the same time to limit as much as possible the diagnostic drift.

Concerning statistical analysis of the data, it should be known that ordinal scores are inherently discontinuous data that are not normally distributed (bell-shaped) and, therefore, they do not meet the assumption of parametric statistical analysis and non-parametric analyses (e.g., Mann Whitney test) should be applied.

4.4. Validation

The MSSS, derived from our experience in fish histology, aims to assess the condition of fish larvae/juveniles, including comparing them according to specific experimental variables. Now, the MSSS has been validated within PFF project activities (Horizon2020). Specifically, a total of 2791 larvae and juveniles (27 GSB batches and 7 ESB batches), obtained over 2 production seasons from 3 European hatcheries, were sampled, and evaluated by the MSSS at different stages. This evaluation process gave more than 60,000 scores; these biological data allowed statistical differences in CHI and OCV to be detected between different batches of fish within the same hatchery or between different hatcheries (data not yet published). Our ultimate project goal was to identify histological indicators of fish health status to support fish farmers in different rearing strategies for welfare and product quality. In addition, among the activities of the PFF, the MSSS was also applied during a thermal imprinting pilot test to evaluate the effect of 3 different temperature regimes applied during the autotrophic phase of GSB and ESB on the phenotype plasticity (results not yet published). The MSSS was tested under experimental conditions to reveal potential histopathological deviations attributable to different water temperature regimes.

Finally, both intra-observer repeatability (same person scoring the descriptors) and inter-observer repeatability (two persons scoring the descriptors) were validated, although currently only within our research group.

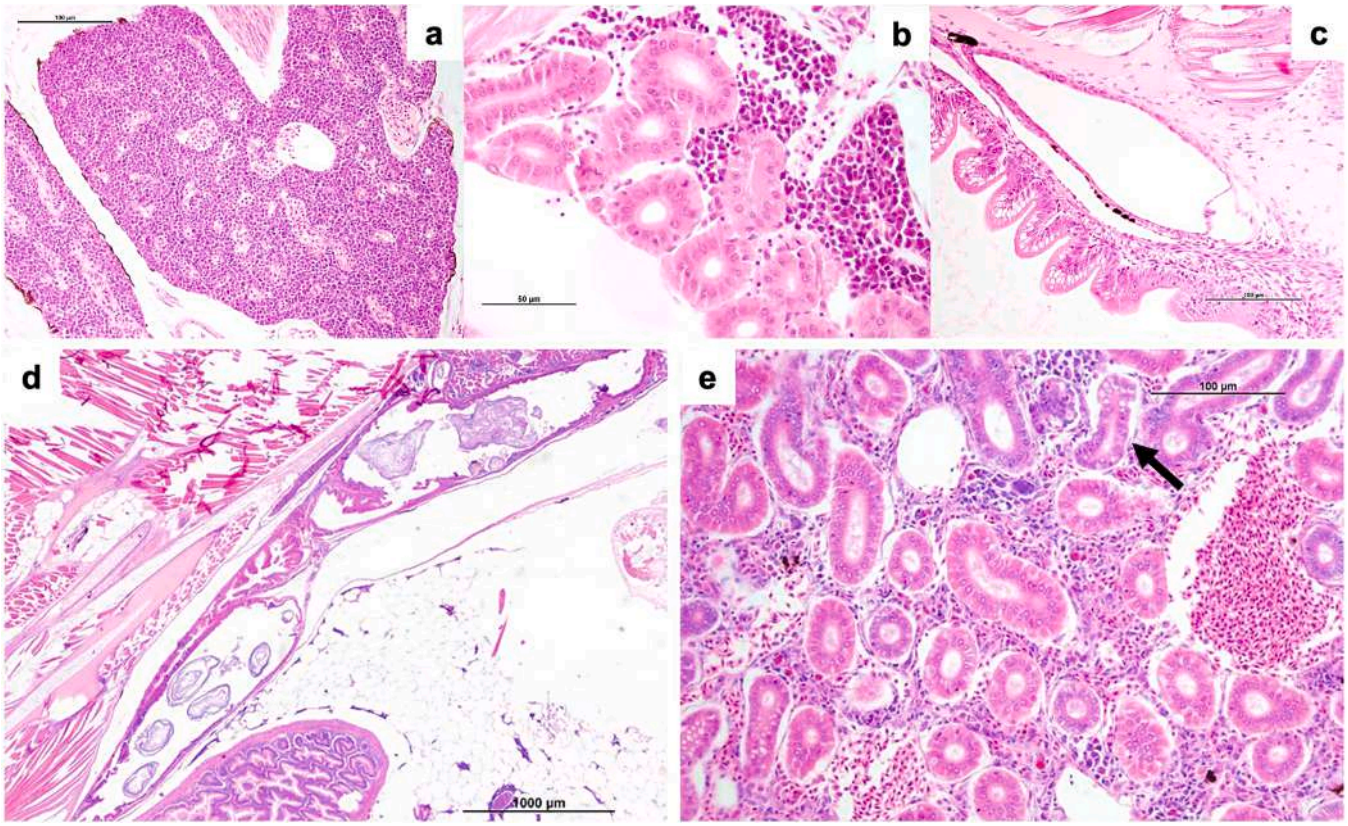

Fig. 6. GSB excretory system descriptors. a) 92 dph, normal kidney, hematopoietic tissue located in the head kidney, HE; b) 92 dph, normal kidney, median portion, renal tubules surrounded by hematopoietic tissue, HE; c) 92 dph, normal urinary bladder HE; d) 95 dph, nephrocalcinosis, presence of large calculi in ureter and urinary bladder, provoking severe alteration in kidney structure, HE; e) 92 dph, presence of inflammatory cells (probably lymphocytes and acidophilic granulocytes with a slight EGC component) in renal hematopoietic tissue, some tubules have mild vacuolar degeneration (arrow), HE.

5. Conclusions

The hatchery phase still represents a bottleneck in marine fish rearing and one of the aims of the H2020 PFF project is to identify quality markers to ESB and GSB larvae and juveniles. Histology represents a useful tool in order to early recognize factors that can limit production. Indeed, each biotic and abiotic factor and each management decision can positively or negatively influence the fish health condition. The original histological Multiparametric Semi-quantitative Scoring System proposed has been developed to investigate on histological hallmarks of marine fish larvae/juvenile batches. Unlike descriptive histology, histological grading system, as well as morphometry, leads to a standardized quantification and allows the possibility of legitimate comparison between different studies (Bernet et al., 1999). Morphometric analysis certainly offers many advantages, in fact it is a robust, reproducible, and rapid method which can be employed for a large number of samples. However, tissue quality of the sample must be adequate (i.e., not too fragmented or degraded by fixation/processing procedures) for accurate performance of quantitative morphometry; the image analysis systems cannot ignore or compensate for those irregularities so easily, while a trained histopathologist is able to do this when the sample is not completely good (Silva et al., 2015) and is able to interpret the data. Moreover, morphometric analysis is more expensive, as the cost of the initial investment in hardware and advanced software for image analysis is still high and must to be included in the cost of the diagnostic service. These reasons lead to the choice of adopting a semi-quantitative method for the purpose of the project, instead of a descriptive or quantitative one. In fact, the MSSS is not excessively expensive as it only requires an image analysis software for basic research and it is applicable on samples coming from commercial fish farms, sampled in sub-optimal conditions by non-specialized operators. The semi-quantitative scoring has the characteristic of being dependent on the observer eye; this can involuntarily cause the presence of bias and poor reproducibility. In this light, the MSSS was developed by overcoming these problems as much as possible, applying blind assessment of samples, detailed criteria for scoring descriptors, repeatability of scoring between observers and an adequate number of fish assessed. Six organs were included in the MSSS; each of them is evaluated by scoring from 1 to 5 specific descriptors. In addition, to avoid the loss of important information, it has been chosen to also evaluate the general architecture of the organs which includes all regressive and progressive alterations. According to the authors, evaluating the organ architecture it is important for understanding the effect that the descriptor alterations and other impairments had on the organ condition. The MSSS provides 24 data per age and batch (18 descriptors plus 6 architecture data) that can be exploited according to the purpose of the survey. An algorithm allows to summarize all the descriptors in a single number (Cumulative Histologic Index – CHI); having a single value indicating the histological status of a batch is useful to obtain a first glance on the status of the batch itself. The CHI alone does not give information on which organ district is most affected by the problem, but when higher than 3.5, it suggests that something is going wrong. In fact, the threshold was specifically set at a very unbalanced value downwards, compared to the upper limit of 25, so that it acts as a warning bell for the fish farmer when the situation is still solvable. The limit of 25 is only virtually achievable, since, to obtain it, all organic districts should obtain 5 both in all descriptors and in architecture. A similar situation is likely to be incompatible with life. On the other hand, specific information is given by the OCV (Organ Condition Value) which shows the general condition of each organ and by the individual descriptors, which also provide a way to understand what is specifically causing the problem. The MSSS outcomes, CHI, OCV and descriptor scores can be used together or individually, to observe the variation between different groups, along with environmental variation and variation in hatchery operating procedures or through the stages of development. The authors underline that histology provides indications of the fish morphological condition in a precise instant of their life history and, therefore, it is not possible to obtain predictive information of the fish quality at the end of the production cycle. Furthermore, fish larval and juvenile stages have an extraordinary growth and recovery potential which is also expressed as high competence in repairing or regenerating (process by which damaged or lost structures are perfectly or near-perfectly replaced) tissues due to pathological events (infectious and non-infectious) (Poss et al., 2003; Beraldo and Canavese, 2011; Zupanc and Sîrbulescu, 2012;

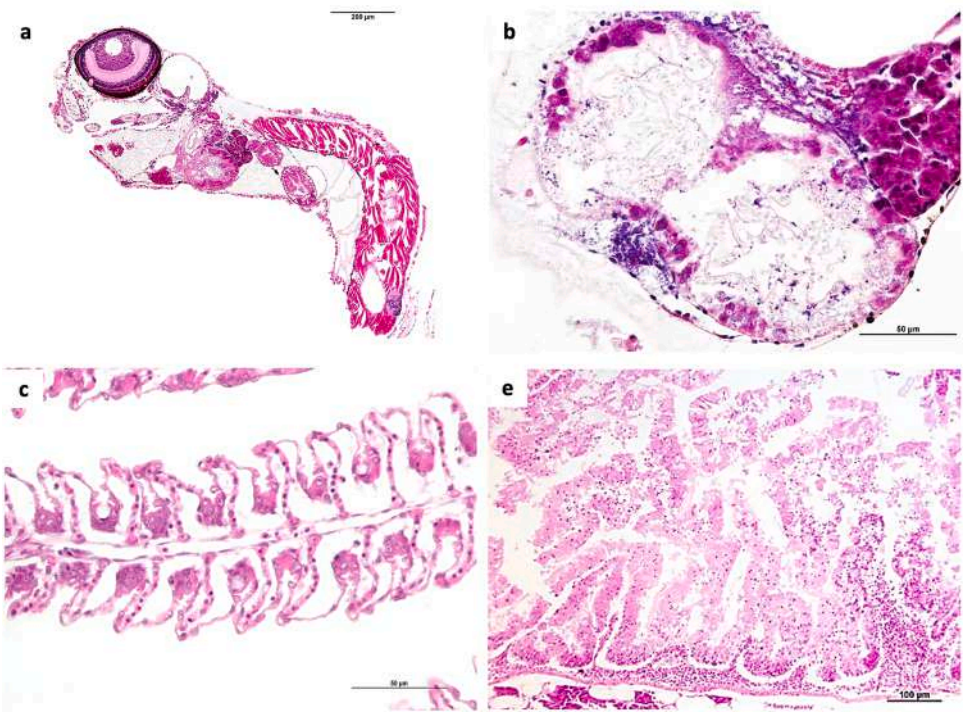

Fig. 7. GSB, examples of inadequate fixed tissue. a) 5 dph, inadequate fixation of a whole larva. Loss of architecture and cellular details in all the larval organs. This condition could be due to a long permanence in fixative solution, HE; b) 5 dph, artifacts in anterior intestine and liver. A massive autolysis of anterior intestine wall is present, this is probably due to a long time from death to fixation, HE; - c) 92 dph, artifact in a gill filament mimics gill edema. The observation of the general fixation condition of the fish leaded to consider this finding as autolyzed tissue, HE - d) 92 dph, inadequate intestine fixation. Autolysis of a large portion of the intestinal wall with a serious loss of the anterior intestinal mucosae architecture, HE.

Choi et al., 2015; Wolf et al., 2015; Bates et al., 2018). Do not take into account this fish abilities could induce investigators to overestimate the long-term impacts of certain lesions on the health of individual fish and fish populations (Wolf et al., 2015). Hence, MSSS cannot be used for a predictive long-term quality evaluation but rather it could be effective in the early highlighting of health problems in a batch, in providing farmers with information about the impact of their rearing practices and, therefore, in helping them to solve critical problems in the hatchery phase. Therefore, it could potentially be a supportive tool both for hatcheries, as internal quality control at different developmental stages, and for on-growing farms for the control of incoming fry. The MSSS could ultimately be a valid tool for the comparison of fish batch quality under different rearing conditions.

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CRediT authorship contribution statement

Valentina Pacorig: Conceptualization, Methodology, Writing – original draft. **Marco Galeotti:** Funding acquisition, Resources. **Paola Beraldo:** Conceptualization, Methodology, Writing – review & editing.

Declaration of Competing Interest

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

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