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Review article

# HISTOLOGICAL METHODS IN THE ASSESSMENT OF DIFFERENT FEED EFFECTS ON LIVER AND INTESTINE OF FISH

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**Abstract:** This manuscript describes the importance of using histological methods to assess the effects of feed on the liver and intestine of fish. Due to the constantly increasing world production of fish and other aquatic organisms, it is necessary to replace fishmeal and fish oil in diets with less expensive raw materials of plant origin. Due to the increased fiber content, increased presence of carbohydrates, antinutritional factors, and inappropriate content of amino acids and new compounds can have negative effects on the digestive system of fish and therefore on fitness, health and production characteristics of cultivated fish. The liver and intestines are the most important organs for the digestion and absorption of nutrients from feed. Therefore, monitoring histological structure of fish liver and intestine is the method of choice in assessing the effects of nutrient mixtures that use raw materials of plant origin. For both of these vital organs the normal histological structure and the most important results obtained by research are discussed. This paper presents a critical review of the histological methods used in research on feed effects. Results related to the negative effects of raw soy-based feed on the occurrence of enteritis in carnivorous fish species are discussed. The results point out that use of modern approach in fish pathology such as improved histochemical, stereological, scoring, and other analytical methods could be a beneficial approach in an accurate assessment of new feed effects on fish.

**Key words:** fish, nutrition, effects, histology, liver, intestine.

## Introduction

To keep fish industry sustainable it is necessary to find an alternative to the basic ingredients for the production of fish food, fish oil and fishmeal. Hardy (2010) argues that the industry will soon run out of sufficient quantities of fish oil and meal. Fishmeal is the best source of protein for fish, because it has a high level of protein, excellent amino acid composition and good digestibility (Watanabe,

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2002; Gatlin et al., 2007). The aim of the fish feed production is the replacement of fishmeal with less expensive source of protein, usually of plant origin. A low level of fibres, carbohydrates and indigestible antinutrients, high protein level, good amino acid profile, high digestibility, and good palatability are appropriate characteristics of good plant ingredients in fish feed (Francis et al., 2001; Marković et al., 2009). Even if all these features are present in plant, it does not mean that it can be used as the complete replacement of fishmeal. It is common that only a part of the proteins in feed is replaced, but even such low level of replacement can have an effect on fish organism, primarily on the digestive tract.

Histopathology together with other methods such as: biochemical, growth, diseases diagnostic, are biomarkers used in assessing effects of both internal (feed used) and external (aquatic) environmental conditions (O'Connell, 1976; Hinton et al., 1987; Segner and Braunbeck, 1988; Tacon, 1992; Poleksić and Mitrović-Tutundžić, 1994; Baeverfjord and Krogdahl, 1996; McFadzen et al., 1997; Poleksić and Karan, 1998; Fanta et al., 2003; Van der Oost et al., 2003; Zimmerli et al., 2007; Grund et al., 2010; Poleksić et al., 2010; Rajeshkumar and Munuswamy, 2011).

Histological analysis of the digestive system is considered a good indicator of the nutritional status of fish (Hall and Bellwood, 1995; Green and McCornick, 1999; Caballero et al., 2003). The intestine and liver are the most important organs in digestion and absorption of nutrients from food, and therefore monitoring of these organs is considered necessary (Takashima and Hibiya, 1982; Roberts, 1989). For this, various methods of histological analysis are used, most often semiquantitative scoring system, histochemical and immunohistochemical method, while stereological methods are rarely used (Rocha et al., 1997; Mouton, 2002).

In this paper, a review of histological methods used in the assessment of effects of different fish feed on the liver and intestine is presented. Semiquantitative, morphometric, stereological and histochemical methods and techniques in histology are considered. The significance of different methods is discussed and evaluated in the light of literature data and results obtained in our research.

## Intestine

Histological structure of this part of the digestive system is: mucosa, which consists of the lamina epitelialis (simple columnar and glandular epithelium) and lamina propria (connective tissue); submucosae, consisting of two layers (stratum compactum and stratum granulosum); muscular layer and serosa (Poleksić et al., 2006). This histological structure is not altered even in experiments in which fish were fed food containing heavy metals (Andreozzi et al., 1994; Kruatrachue et al., 2003). Histopathological changes in the intestine may vary depending on the

species and feed used in the experiments. In fish from *Salmonidae* family, the replacement of fishmeal proteins with plant proteins causes reduced growth rates and pathological changes, particularly enteritis in the distal part of the intestine (Urán et al., 2009a). Studies have shown that Atlantic salmon (*Salmo salar* L.) is more susceptible to enteritis than the rainbow trout (*Oncorhynchus mykiss* L.) (Refstie et al., 2000). In fish intestine enteritis represents a set of changes visible at the light microscopy level, described and named by Baeverfjord and Krogdahl (1996) as "non-infectious subacute enteritis": shortening of intestinal villi, loss of supranuclear vacuolization of the enterocytes, widening of lamina propria of villi, and infiltration of inflammatory cells in the lamina propria. Recently, a semiquantitive scoring system for evaluation of intestinal enteritis of the rainbow trout and the Atlantic salmon was proposed by Uran et al. (2008). The scoring system is based on assigning a number, which depends on lesion severity: from 1 (normal histology) to 5 (distinct pathological condition) for changes observed in the intestine, and therefore quantifying the changes found.

Using similar methodologies, two groups of scientists: one from Norway and one from the Netherlands investigate effects of the replacement of fishmeal with proteins of plant origin (mostly soybean meal) in fish feed. Studies showed that enteritis is dependent on the percentage of replacement of fishmeal. First signs of enteritis in rainbow trout are observed when 10% of fishmeal is replaced (Krogdahl et al., 2003). The replacement of 30% and more has been critical for digestion (Baeverfjord and Krogdahl, 1996). Increasing the percentage of soybean meal in feed led to the increased intensity of enteritis (Krogdahl et al., 2003; Urán et al., 2009a). Results showed that after only two (Baeverfjord and Krogdahl, 1996), or three days (Urán et al., 2009a) the first signs of enteritis occur in fish intestine. These changes were observed in fish fed with a higher percentage of soybean meal in feed (20-33%), while fish fed with lower percentage of soybean meal in the feed (10%) showed first signs of enteritis after seven days (Urán et al., 2009a). The other experiment showed that soybean origin has an impact on the intensity of enteritis in Atlantic salmon (Urán et al., 2009b). In this study a soybean of different origin (Europe, South America, North America and Australia) was evaluated, and results clearly show that the origin has an effect on the severity of soybean meal-induced enteritis in Atlantic salmon.

In common carp (*Cyprinus carpio* L.), reaction to the replacement of fishmeal for soybean meal is different compared to the family *Salmonidae*. According to Uran et al. (2008) there is a short-term reaction and inflammation to soybean, but after a period of one month, after adaptation, the intestine returns to a normal histological structure. The carp has a better tolerance to the presence of antinutritive ingredients in food, as Francis et al. (2001) claim that tolerance is specific for the species. In the experiment with Egyptian Sole (*Solea aegyptiaca* Chabanaud) 30% of fishmeal was replaced with soybean meal and no pathological

changes in intestines were observed (Bonaldo et al., 2006). Similar results were found when Grisdale-Helland et al. (2002) performed the experiment with Atlantic halibut (*Hippoglossus hippoglossus* L.), where the percentage of replacement in the diet was 36%, and Nogales Mérida et al. (2010) when 30% of fishmeal was replaced with sunflower meal in sharpsnout seabream (*Dyplodus puntazzo* Cetti). In experiment with sharpsnout seabream, the quantification of histological parameters was carried out by morphometry of the intestinal folds (*villi intestinales*) by the method of Lunstedt et al. (2004). The length of villi, width of the lamina propria, width of the villi, thickness of the muscle layers and the number of goblet cells per segment were quantified. Samples were taken from all three parts of the intestine (proximal, medial and distal).

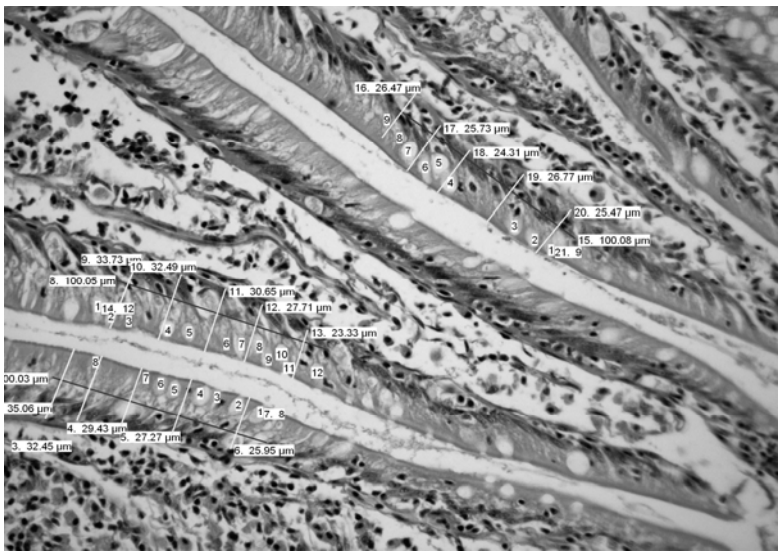


Figure 1. Intestine of experimental carp (HE; x400). Measurement of enterocytes height is shown. Scale bar = 50 µm.

In our studies that evaluated effects of different fish feed on production traits of the common carp, we did not observe any intestinal changes or other nutritional pathologies. In an experiment conducted using carp in which 30% of fishmeal was replaced with proteins of plant origin (soy, wheat and corn), length of villi and height of enterocytes (Figure 1) were measured to evaluate histological condition of the intestine (Rašković et al., 2009a). Four feed formulations were used in which the protein part (38% of feed) was of different origin. Morphometric parameters showed that the enterocytes height was significantly shorter in the group fed feed in which fishmeal was totally replaced by plant protein (Poleksić et al., 2007).

These results are related to the growth of the fish, as in all experiments in which intestinal pathology was found. Enterocytes height is a useful histological parameter that could be followed in experiments not just related to the replacement of fishmeal, but also in evaluating different types of commercial feed. In the experiment where rainbow trout cultured in cages were fed on the lowest lipid content, they had an increase in enterocyte height, as well as an increase in the number of mucous cells (Poleksić et al., 2006).

Apart from a routine, hematoxylin-eosin staining, histochemical staining techniques are used. If the whole digestive tract is the point of interest, samples are stained by trichrome stains (Humason, 1972; Roberts, 1989; Marchetti et al., 2006; Leknes, 2010). This type of staining demonstrates muscle, collagen fibers, fibrin and erythrocytes (Bancroft and Stevens, 1977). If the point of interest are mucopolysaccharides (mucins and glycopolysaccharides), usually periodic acid-Schiff and Alcian blue techniques are performed. Glycogen and other carbohydrates in tissues are best demonstrated by periodic acid-Schiff reaction, which color carbohydrates to magenta. Alcian blue demonstrates acid mucins in tissue and stains them blue: strong sulphated mucins are colored at pH 0.5, weakly sulphated mucins at pH 2.5, hyaluronic acid and sialidase-labile sialomucins at pH 3.2 and sialidase-resistant sialomucins at pH 1.0 (Bancroft and Stevens, 1977). Histochemical methods can be used for gathering additional parameters, for example, activity of certain enzymes in the intestine. Gawlicka et al. (2002) used histological sections for determining a position on white sturgeon (*Acipenser transmontanus* R.) intestine where activities of nonspecific esterase, alkaline phosphatase, and lipase are present. Bakke-McKellep et al. (2000) evaluated the seriousness of enteritis in the intestine of Atlantic salmon using histochemical methods. The results showed a reduced presence of enzymes that are specific to microvilli and enterocytes.

Stereology is a method of obtaining three-dimensional structures according to the two-dimensional histological sections (Weibel et al., 1966; Mouton, 2002). It represents the most objective method for evaluation of histological samples (Gundersen et al., 1988). Stereological methods are well developed and many studies of intestinal histology of different animals are carried out. Unfortunately, in fish feeding experiments stereological methods are seldom used. Enterocytes are showing relatively limited response and therefore, studies on the ultrastructural level are favored (Olsen et al., 1999, 2000). One of the few stereological studies was done by Wold et al. (2009). Authors evaluated the effects of different types of lipids (phospholipids and neutral lipids) on the intestinal histology of the cod larvae (*Gadus morhua* L.). In this study, a systematic sampling described by Mayhew (1991) and measuring the volume density using Cavalieri's principle (Gundersen et al., 1988; Mayhew, 1991) were used. This approach provides complete objectivity and reduces the possibility of error.

## Liver

A number of authors connect liver morpho-physiological condition with feeding (Takashima and Hibiya, 1982; Roberts, 1989; Caballero et al., 2003; Ostaszewska et al., 2005). The liver is considered as a good indicator of nutritional pathology due to its function in metabolizing products coming from the digestive tract. Fish liver histology is characterized by the absence of liver lobules and portal triads that are the basic morphological unit of liver structure in mammals. Although all structural elements (liver cells-hepatocytes, blood vessels, and bile ducts) are present in the liver of fish, they are differently organized compared with mammals. Bile ducts are isolated or in groups of 2 to 3, often accompanied by arterioles (Rocha et al., 1994). Fish hepatocytes lack organization in Remack cords, comparing to mammals. Instead, fish hepatocytes exhibit a diffuse or radial organization in branching tubules that form liver parenchyma (Rocha et al., 1994).

Histological changes in the liver are easily recognized if the food used is not adequate (Tacon, 1992). The most common changes observed in the liver are: hepatocytes vacuolization, fatty degeneration of the liver, changes in metabolic activity, changes in liver parenchyma and necrosis (Takashima and Hibiya, 1982; Roberts, 1989; Poleksić et al., 1995). If food containing protein or fat is used, this can lead to histopathological changes in the liver (Caballero et al., 2004).

When liver is used as organ of choice in histopathology, difficulties can occur since the liver is very sensitive to any source of pollution (Hinton et al., 1987). This is important to take into account when aquaculture production experiments are performed. Another aspect that should be taken into consideration is the age of fish. Segner and Juario (1986) published results pointing to different feed requirements before and after metamorphosis in liver of milkfish (*Chanos chanos*, Forsskål). They followed liver histology of milkfish larvae from day 10 to day 35 after hatching, and fed fish 6 different diets. They have established that feed containing live food, in this case (*Artemia nauplii*), showed better histological structure. But during metamorphosis, even natural food caused mild degenerative changes.

As indicator of the metabolic activity of hepatocytes, morphometric parameters are most often used: hepatocytes number, hepatocytes surface area, hepatocytes nuclear area, and glycogen and lipid content in the cytoplasm (Strüssmann and Takashima, 1990). By the use of mentioned parameters it is possible to compare the liver status of animals in the treatment. The method was used by Zakes et al. (2008): extracts of two medicinal plants *Astragalus radix* and *Lonicera japonica* were added in fish feed. Negative effects of these plants on the liver structure (increased necrosis and liver vacuolization), and no effect on growth were observed. Similar method was used by Ostaszewska et al. (2005), in the study of the effects of casein replacement in feed by soy on the rainbow trout and pacu

(*Piaractus mesopotamicus*). A difference between the two species was demonstrated: pacu hepatocytes had normal structure, while rainbow trout hepatocytes had picnotic nuclei of irregular shape and their position in cell was excentric. In another paper Ostaszewska et al. (2008) have shown a positive effect of natural food on hepatocytes structure of the silver bream (*Vimba vimba*), as well as approximately the same ratio of lipid and glycogen in liver cells. Yamamoto et al. (2008) used liver cytoplasm and nucleus diameter of rainbow trout to examine the effect of a supplemental ethanol extract from a defatted soybean meal on liver. The results showed that fish fed ethanol extract had atrophied hepatocytes. Russell et al. (2001) fed European sea bass (*Dicentrarchus labrax* L.) experimental diets with a varying starch source (pea and corn starch). This resulted in sinusoidal reduction in fish fed the pea meal diets and a more uniform appearance in the hepatocyte distribution. They also showed an increase in glycogen deposition in livers of sea bass fed the pea meal diets.

Our experiments have demonstrated that liver histology is inevitable in nutrition studies (Poleksić et al., 2007; Rašković et al., 2009a). Similarly, our studies have shown that hepatocytes nuclear area measurement can be a useful parameter in this type of investigation (Figure 2).

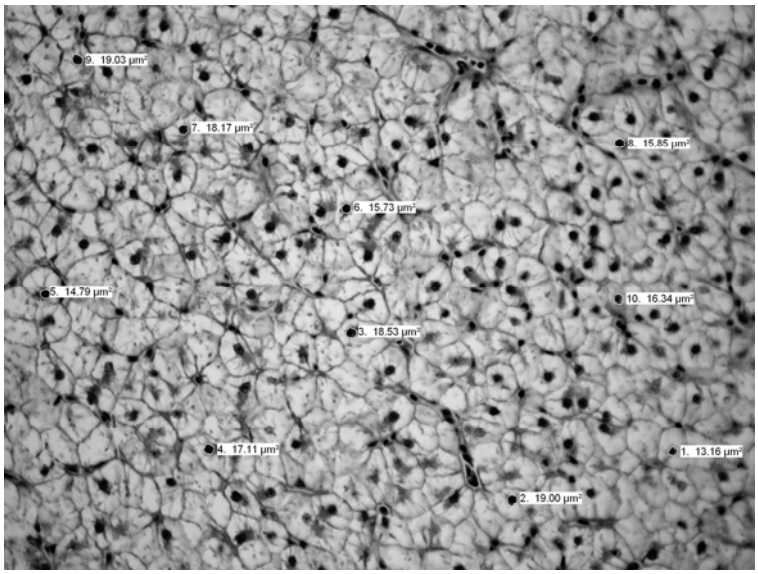


Figure 2. Hepatocytes of experimental carp (HE; x400). Measurement of nuclear area is shown. Scale bar = 50 µm.

A size of a nucleus reveals changes in liver metabolism, such as picnosis, kariolysis of the nucleus or necrosis of the cell. In laboratory experiments where

fishmeal was replaced with soybean meal, a piknosis of liver nuclei is observed, as well as smaller nuclear area. These findings point at malnutrition of fish fed feed where soybean meal is the only protein source. In field experiments, we have found a clear season-dependence of average hepatocyte nuclear area in the liver of rainbow trout reared in cages (Poleksić et al., 2006; Savić et al., 2008; Rašković et al., 2009b).

The mentioned methods are quantitative. Semiquantitative methods are found in the literature, such as the method suggested by O'Connell (1976): changes of hepatocytes of the northern anchovy (*Engraulis mordax* Girard) during starvation were assessed according to three criteria: changes in the nucleus, changes in the cytoplasm, hepatocytes vacuolization. The method is semiquantitative since the level (degree) of hepatocytes alteration is described by a number attributed: 1 (normal histology) to 3 (marked histological changes). A modified system was used by McFadzen et al. (1997) in the study of nutritional condition on *Sardina pilchardus* Walbaum larvae.

There are more stereological studies concerning effects of feed on fish liver than those on the intestine. Nogales Mérida et al. (2010) counted liver cells to investigate effects of replacement of fishmeal with sunflower meal on the liver. The difference in hepatocytes count was not statistically significant. A good example of use of both stereological and semiquantitative methods is the study of Caballero et al. (2004): sea bream (*Sparus aurata* L.) was fed different kinds of vegetable oils as replacement for fish oil, and effects on liver were monitored. Besides semiquantitative assessment of the liver changes that consisted of the evaluation of histological changes (nuclear displacement and cytoplasm vacuolization) with four scores: 0-not observed; 1-few; 2-medium; 3-severe; a point counting method for determination of volume of hepatic lipid in liver was used. As already mentioned, Wold et al. (2009) used stereological methods to find out that cell and hepatocytes nuclei are smaller in cod fed neutral lipids. In addition, these authors concluded that more lipids are stored in hepatocytes of fish fed feed containing phospholipids. Verreth et al. (1994) have demonstrated liver changes in African sharptooth catfish (*Clarias gariepinus* Burchell) fed Artemia containing lower (coconut oil) and higher (SELCO) level of highly unsaturated fatty acids.

## Conclusion

The use of different methods in the evaluation of the effects of feed on intestinal and liver histology is a must. Methods of choice in many studies are semiquantitative methods, since they are not time-consuming, and are easy for evaluation of samples, but they are not objective as others. Morphometric methods are more relevant than semiquantitative, but the samples are often not collected

systematically, which could lead to an error. Therefore, stereological methods are recommended in histological evaluation of samples. These methods are rarely used, because the scientists need to have advanced knowledge of this area. Nevertheless, all those methods are complementary to other results obtained in experiments and can provide additional information not only of morphological state of the organ, but they can also contribute to better explanation of mechanisms involved in weight gain, digestibility and food availability.

In future, experiments in the area of fish nutrition, histological state of liver and intestine should always be taken into account. This should provide additional information about state of these organs if any of mentioned methods are used. These methods are valuable in field experiments, as well as in laboratory. They can show signs of malnutrition, starvation, or negative effect of feed on liver and intestinal morphology. Nutritional pathology can explain lower production results in fish fed inadequate feed. They are also able to show an influence of the environment on a delicate structure of liver and intestine, most often of subacute pollution in fish ponds.

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# HISTOLOŠKE METODE U PROCENI EFEKATA HRANE NA MORFOLOGIJU CREVA I JETRE RIBA

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R e z i m e

U radu je prikazan značaj upotrebe histoloških metoda u proceni efekata hrane na jetru i crevo riba. Usled konstantno rastuće svetske proizvodnje riba i drugih vodenih organizama pokazalo se neophodnim da se riblje brašno i riblje ulje u hranljivim smešama sve više zamenjuju manje skupim sirovinama biljnog porekla. Zbog povećanog sadržaja vlakana, povećanog prisustva ugljenih hidrata i antinutricionih faktora, te neodgovarajućeg sadržaja amino kiselina u ovim sirovinama delovanje novih smeša može negativno da utiče na digestivni sistem riba a time i na kondiciju, zdravstveno stanje i proizvodne karakteristike gajenih riba. Budući da su jetra i creva najvažniji organi u procesu varenja i apsorpcije hranljivih materija iz hrane, praćenje histološke građe jetre i creva riba je metoda izbora pri proceni efekata hranljivih smeša u kojima se koriste sirovine biljnog porekla. Za oba pomenuta vitalna organa je prikazana normalna histološka građa i najvažniji rezultati dobijeni u istraživanjima autora i iz literaturnih izvora. U radu je dat kritički osvrt na histološke metode koje se koriste u ovoj vrsti istraživanja i na najvažnije dobijene rezultate. Posebna pažnja je posvećena negativnom delovanju sirovina na bazi soje koje uzrokuju pojavu enteritisa kod karnivornih vrsta riba. Ukazuje se i na mogućnosti unapređenja metodologije procene efekata hrane korišćenjem savremenih pristupa u patohistološkim istraživanjima kao što su unapređene histohemijske, stereološke i druge metode.

**Ključne reči:** ribe, ishrana, efekti, histologija, jetra, crevo.

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