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PATHOMORPHOLOGICAL AND BIOCHEMICAL STUDY OF THE GOLDEN GREY MULLET *CHELON AURATUS* (RISSO, 1810) IN THE WATERS OF THE SOUTHWESTERN CRIMEA (THE BLACK SEA)

© 2022 **T. V. Gavruseva, T. B. Sigacheva, and I. I. Chesnokova**

A. O. Kovalevsky Institute of Biology of the Southern Seas of RAS, Sevastopol, Russian Federation
E-mail: *gavrt2004@mail.ru*

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The golden grey mullet *Chelon auratus* (Risso, 1810) (Mugilidae) is a valuable commercial and recreational species ranking first in terms of catch volume of the Black Sea indigenous mullets. The importance of this species in the regional fishery among demersal fish requires the development of a system for assessing its health status. Such research is based on an integrated approach involving biochemical and pathomorphological methods: these allow to investigate the alterations in fish *prior* the occurrence of visible manifestations, disruption of the processes of growth and reproduction, reduction of commercial size, and decrease in abundance. The aim of our work was to study both pathomorphological alterations and several biochemical parameters of golden grey mullet tissues for assessing its health status. Fish visual examination and pathological autopsy were carried out. For histological analysis, samples of the gills, liver, kidneys, gastrointestinal tract, spleen, and pancreas were fixed in Davidson's solution and processed by standard methods. Based on the histological studies, the fish health status was investigated by a modified semi-quantitative analysis of alterations according to the Bernet *et al.* protocol and by assessing the distribution of lesion in organs using a scoring system. We determined the importance factors of alterations for *C. auratus*, the values of organ alteration indices, and the total index of fish pathology. The biochemical studies permitted to reveal the level of protein oxidation, lipid and urea peroxidation, and the activity of aminotransferases and alkaline phosphatase in the liver; moreover, we quantified albumin and glucose concentration in the blood serum. In the organs of the golden grey mullet, the histopathological alterations referring to four types of the reaction patterns were detected (circulatory disorders, regressive and progressive alterations, and inflammatory processes). Furthermore, parasites representing several species of different systematic groups (Protozoa, Monogenea, Trematoda, and Nematoda) were identified. It was established that the most severe histopathological alterations were caused by a parasitic protozoan, presumably *Ichthyophonus* sp. When carrying out a semi-quantitative analysis of alterations, the mullets were conventionally divided into conditionally healthy individuals and infected ones. Pathomorphological data were obtained, and the set of biochemical parameters was compared in these two groups. Significant differences were revealed in the values of organ alteration indices in *C. auratus* in the kidneys, liver, gastrointestinal tract, and pancreas. The values of the total index of fish pathology also differed significantly. The biochemical studies revealed a significant increase in urea content in the liver of fish from the group 2, that may indicate the kidney and gill excretory dysfunction (it was confirmed histologically). No significant differences were found in the level of lipid peroxidation, protein oxidation, and activity of aminotransferases in the liver of conditionally healthy and infected fish. The results of our investigation confirm high informativeness of the studied parameters for assessing the health status of the golden grey mullet.

Keywords: golden grey mullet, histopathological alterations, biochemical parameters, semi-quantitative analysis, Black Sea

The golden grey mullet *Chelon auratus* (Risso, 1810) (Mugilidae) is a valuable commercial and recreational species ranking first among the Black Sea indigenous mullets. It is characterized by a wide geographical distribution and high productivity (Boltachev & Karpova, 2012 ; Kozhurin et al., 2018). In commercial catches off the Crimean coast, the species constitutes about 95 %; *Mugil cephalus*, less than 5 %; and *Chelon saliens*, less than 1 %. In 2000–2017, according to the literature data, the interannual dynamics of Mugilidae catch in the Black Sea was characterized by a positive trend in 2000–2007, a decline in annual catches in 2008–2010, and a rapid growth in 2011–2017, caused by an increase in the Crimean fish stocks. Specifically, Mugilidae annual catch in 2000 was 18.8 tons; in 2017, it was 275.4 tons, almost 15 times higher (Kozhurin et al., 2018).

In the regional fishery management, high importance of the golden grey mullet among demersal fish requires the development of a system for assessing its health status. The world experience in carrying out this kind of research is based on the integrated approach involving biochemical and pathomorphological methods (Kornienko et al., 2018 ; Lukina, 2014 ; Kundu et al., 2016 ; Osman et al., 2009). These allow to study the alterations in fish (resulting from parasitic invasions and negative effect of the environment) *prior* to the occurrence of visible manifestations, disruption of the processes of growth and reproduction, reduction of commercial size, and decrease in abundance.

Considering the key role of free radical processes in the mechanisms of formation of pathological alterations in the fish, it is recommended to assess the health of hydrobionts based on biochemical parameters of tissue damage under oxidative stress [levels of lipid peroxidation and protein oxidation (hereinafter LPO and PO, respectively)] (Lukina, 2014 ; Kurhalyuk & Tkachenko, 2011 ; Marcogliese et al., 2005) and biomarkers of fish physiological state in whole (activity of aminotransferases and alkaline phosphatase (hereinafter ALP), as well as urea, glucose, and albumin content) (Feist et al., 2015 ; Nnabuchi et al., 2015 ; Noor et al., 2010 ; Osman et al., 2009). Moreover, to assess the fish health status, methods of clinical and pathological examination are applied (Moiseenko et al., 2010 ; Frasca et al., 2018 ; ICES, 2015). The most widely used parameters are skeletal deformities, fin erosion, epidermal hyperplasia, and pathological alterations in internal organs (haemorrhagia, tumor, *etc.*) (Moiseenko et al., 2010 ; Au, 2004 ; Frasca et al., 2018 ; Stentiford et al., 2009).

Methods of the histological study allow to reveal the initial stages of pathological disorders in organs and tissues, which cannot be detected by visual examination. The applying of the methods of modern histochemistry helps in assessing the functioning features of various tissue and cellular structures, in determining the nature and rate of metabolic processes, and in detecting pathogenic agents in fish organs (Bruno et al., 2006 ; Frasca et al., 2018 ; Noga, 2010). Several authors have attempted to develop a system for semi-quantitative assessment of histopathological features (Bernet et al., 1999 ; Costa et al., 2009 ; Saraiva et al., 2015). The most used one is a semi-quantitative scoring system in accordance to Bernet *et al.* (1999), which is based on the assumption that histopathological alterations have different effect on fish organs (they are of different relative importance or severity). Using a numerical value to the relative importance of the alteration and a degree of its prevalence, the index of the histopathological state of each individual is obtained (Bernet et al., 1999 ; Costa et al., 2009 ; Saleh & Marie, 2016).

The helminth fauna of the golden grey mullet in the Black Sea has been described quite fully, and the localization of parasites has been determined (Dmitrieva & Gaevskaya, 2001 ; Dmitrieva & Gerasev, 1996 ; Pronkina & Belofastova, 2005 ; Yurakhno, 2009 ; Yurakhno & Ovcharenko, 2014). However, the data on the effect of pathogenic agents on biochemical processes and the state of tissues and organs in this fish species are quite scarce (Öztürk, 2013).

The aim of this work was to study the pathomorphological alterations in combination with several biochemical parameters of the liver and blood of the golden grey mullet to assess its health status. In this regard, the following objectives were defined: to investigate the histopathological alterations in juvenile mullets; to carry out a gradation of the revealed alterations and their semi-quantitative analysis; to study the set of biochemical parameters in the liver and blood serum of the individuals investigated; and to determine the informativeness of applying the semi-quantitative analysis of histopathological alterations and the set of biochemical parameters for assessing the health status of the golden grey mullet.

MATERIAL AND METHODS

The object of the study was the Black Sea golden grey mullet *Chelon auratus* (Risso, 1810) (Pisces: Mugilidae) sampled in February 2018 in the Matyushenko Bay (44°37′576″N, 33°31′515″E, Sevastopol). The fish were subjected to a standard biological analysis to determine key linear and weight characteristics. Histological and biochemical studies were carried out on a unified sample of juvenile fish specimens: TL 12.6–19.7 cm; $TL_{average}$ (16.8 ± 3.99) cm; 2 years. For biochemical and pathomorphological analysis, tissues were resected within the first hour after catching fish, *i. e.* the tissues of live mullets were used. When examining the individuals for external or internal alterations, the presence of clinical signs of pathology was recorded (Moiseenko et al., 2010 ; Frasca et al., 2018). During visual examination and autopsy, the calculation of the incidence of alterations was carried out on the entire sample (78 specimens). Only alive individuals (33 specimens) were subjected to histological and biochemical analysis. The fish were preliminarily "euthanized" by adding benzocaine (0.4 g *per* 10 L) to the aquarium (Zav'yalova et al., 2012); the golden gray mullets were left in the solution for at least 10 minutes after cessation of movement.

For histological and histochemical analysis, the fish were fixed in Davidson's solution. Further processing of histological samples and staining of preparations with hematoxylin-eosin according to Meyer, Romanowsky–Giemsa, Ziehl–Neelsen, and Gram were carried out by generally accepted methods (Bancroft et al., 1990). The pathogenic agents detected in the tissues and organs of the golden grey mullet were determined in histological sections based on the results of the histochemical research and considering peculiarities of various classes of parasites (Gaevskaya, 2004 ; Bruno et al., 2006 ; Floyd-Rump et al., 2017 ; Noga, 2010). Since the symptoms of ichthyophonosis are very similar to pathological alterations in fish with tuberculosis caused by acid-fast bacilli and microsporidia, the sections were stained according to Gram and Ziehl–Neelsen to detect these microorganisms (Bruno et al., 2006 ; Noga, 2010).

Histopathological alterations were assessed according to four types of the reaction patterns (circulatory disorders, regressive and progressive alterations, and inflammatory processes); pathogenic agents were taken into account as well (Bernet et al., 1999 ; Costa et al., 2009 ; Santos et al., 2014 ; Saraiva et al., 2015). Each type of the reaction patterns included several alterations that affected either organ functional units or the entire organ. Three degrees of significance (severity) of histopathological alteration were established (importance factors): 1, minimum pathological significance, when the organ damage is easily reversible; 2, moderate pathological significance, the organ damage is reversible in most cases if the stress factor is neutralized; and 3, severe pathological significance, the organ damage is usually irreversible, which results in partial or complete loss of the organ function (Bernet et al., 1999).

To assess the distribution of lesion in organs, a scoring system was used with the following scores: 0, absent or normal; 1, low (≤ 20 %); 2, moderate (21–40 %); 3, often (41–60 %); 4, very often (61–80 %); and 5, diffuse distribution (81–100 %).

Applying the importance factor and the score, the organ alteration index (I_{org}) was determined (Bernet et al., 1999):

$$I_{org} = \Sigma_{rp}\Sigma_{alt}(a_{org} \times w_{org\,rp\,alt}) , \quad (1)$$

where org denotes an organ;

rp, a reaction pattern;

alt, an alteration;

a, a score;

w, an importance factor.

The higher the index, the more the distribution of lesion.

To compare the general health status of the studied individuals based on the revealed histological disorders, the total index of fish pathology (IT) was also quantified (Bernet et al., 1999):

$$IT = I_g + I_k + I_l + I_{gt} + I_p + I_s , \quad (2)$$

where I_g, I_k, I_l, I_{gt}, I_p, and I_s denote the indices of the gills, kidneys, liver, gastrointestinal tract, pancreas, and spleen, respectively.

When analyzing IT values, the fish were conventionally divided into two groups: conditionally healthy individuals and infected ones. Comparative analysis of pathomorphological data and the set of biochemical parameters was carried out between these two groups.

For the biochemical studies, the liver and blood serum of the golden grey mullet were used. To obtain the supernatant, the liver was repeatedly washed with cold 0.85 % saline, homogenized, and centrifuged (10,000 g) for 15 minutes. The fish were bled from the tail vein. The serum was obtained by keeping in the cold. In the liver supernatant, the content of oxidized proteins (optical units·mg^{-1} protein) was determined by the reaction of interaction of oxidized amino acid residues of proteins and 2,4-dinitrophenylhydrazine. The derivatives of 2,4-dinitrophenylhydrazone resulting from this reaction were recorded at the following wavelengths (λ): at 356 and 370 nm, aldehyde (C_{356}) and ketone (C_{370}) neutral products; at 430 and 530 nm, aldehyde (C_{430}) and ketone (C_{530}) basic products (Dubinina et al., 1995).

The content of thiobarbituric acid reactive substance (hereinafter TBARS; nmol TBA·mg^{-1} protein) in fish liver was determined by the reaction with thiobarbituric acid (Stal'naya & Garishvili, 1977). Using Olvex Diagnosticum standard reagent kits (Russia), the activity of aspartate aminotransferase (hereinafter AST; μmol·h^{-1}·mg^{-1} protein), alanine aminotransferase (hereinafter ALT; μmol·h^{-1}·mg^{-1} protein), and ALP (nmol·sec^{-1}·mg^{-1} protein) was determined; urea content (mmol·g^{-1} wet tissue) in liver supernatants was quantified; and concentration of total protein (mg·mL^{-1}), albumin (mg·mL^{-1}), and glucose (mmol·L^{-1}) in the fish blood serum was accessed.

The analysis was performed on a SF-2000 spectrophotometer (OKB Spektr, Saint Petersburg, Russia). The values of the biochemical parameters of liver supernatants were recalculated *per* mg of protein in a wet tissue weight, with concentration by using the Olvex Diagnosticum standard reagent kits.

The results were processed statistically; the arithmetic mean and standard error were calculated ($M \pm m$). The normality of the distribution of the sample was checked applying the Shapiro–Wilk W-test. Differences between the samples were compared by the Mann–Whitney U-test. Differences were considered significant at $p \leq 0.05$. Statistical analysis was performed using PAST 3 and Microsoft Excel 2016 software.

RESULTS

Visual examination. No clinical signs of pathology were revealed. During visual examination, pathological lesions (small white inclusions) were observed in the gills and gill cavity in 2.56 % of the golden grey mullet. During autopsy, nematode larvae were found in the body cavity in 7.69 % of the fish; their livers were greenish (1.28 %), and the spleens were with dark dots (1.28 %).

Histological studies. Several types of alterations were revealed. In particular, in the gills, local necrosis, hyperplasia of the respiratory epithelium, and adhesion of several gill lamellae were recorded (Fig. 1). On gill lamellae, single monogenean parasites (Fig. 1A) and ciliated *Trichodina* sp. (Fig. 1B) were detected; protozoan cysts were found in gill filaments.

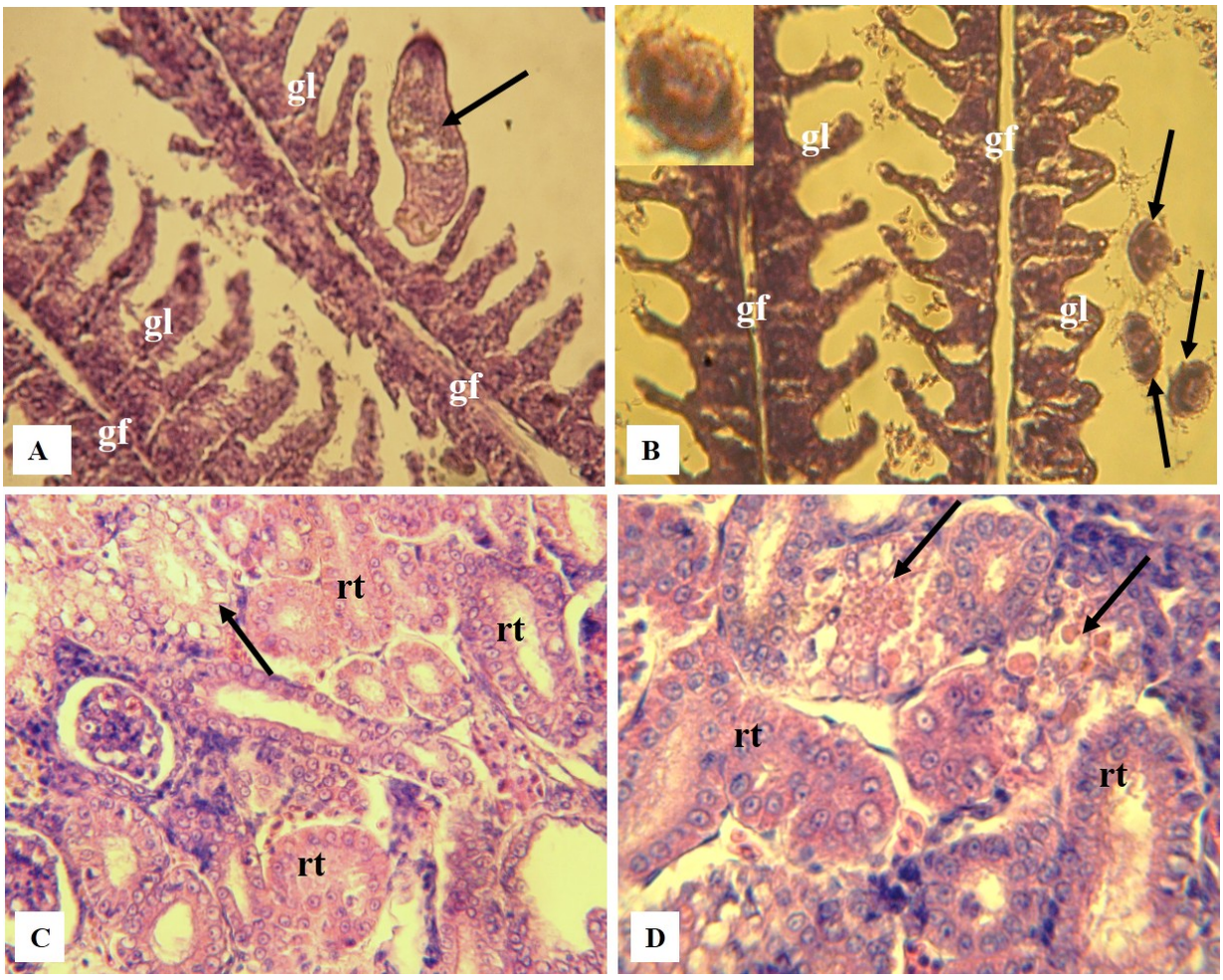

Fig. 1. Histopathological alterations in the gills and kidneys of the golden grey mullet: A, necrosis, hyperplasia of the respiratory epithelium of gill lamellae, and monogenean parasite (↑); B, adhesion of gill lamellae and trichodines (↑); C, local vacuolization of the renal tubule epithelium (↑); D, hyaline droplet degeneration and necrosis of nephrocytes (↑) (×400, hematoxylin-eosin). Gf denotes gill filaments; gl, gill lamellae; rt, renal tubules

Local vacuolization (Fig. 1C), hyaline droplet degeneration and necrosis of nephrocytes (Fig. 1D), and initial renal tubular nephrocalcinosis was observed in the kidney. In the lumen of the renal tubules, an accumulation of plasmodia of protozoan parasites (microsporidia/myxosporidia) was found.

In the liver parenchyma, a slight inflammatory reaction around the blood vessels and bile ducts (Fig. 2A) was revealed. Vacuolization, fatty dystrophy (Fig. 2B), nuclear pleomorphism, and necrosis of several hepatocytes (Fig. 2C) were registered. Moreover, in fish liver, spleen, pancreas, and hematopoietic tissue of the kidney, deposition of ceroid / melanomacrophage centers (hereinafter MCs) (Fig. 2D) were recorded.

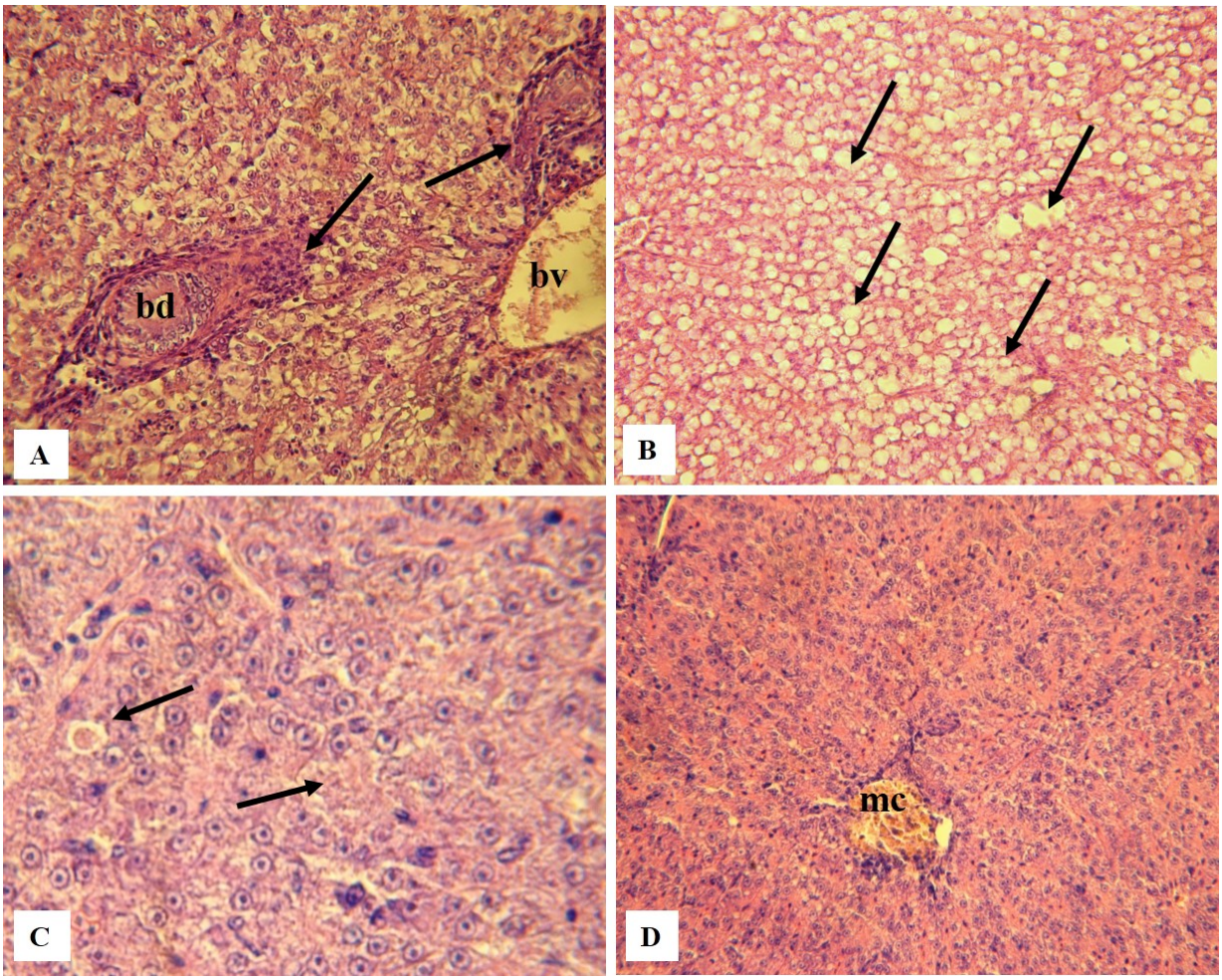

Fig. 2. Histopathological alterations in the liver of the golden grey mullet: A, inflammatory reaction around the bile ducts and blood vessels (↑); B, fatty degeneration of hepatocytes (↑); C, focal necrosis of hepatocytes (↑); D, melanomacrophage center (×400, hematoxylin-eosin). Bd denotes bile duct; bv, blood vessel; mc, melanomacrophage center

The analysis of the pyloric stomach and pyloric caeca revealed local cell necrosis in the mucous layer, as well as edema, hyperemia (Fig. 3A), inflammatory reaction, and protozoan cysts (myxosporidia) in the submucosal layer (Fig. 3B). Nematodes and trematodes were found in the lumen of pyloric caeca (Fig. 3C). In the exocrine pancreas of the infected mullets, steatosis (fatty degeneration of cells), local hyperemia (Fig. 3D), focal cell necrosis, and hemosiderin deposition around nematodes were detected.

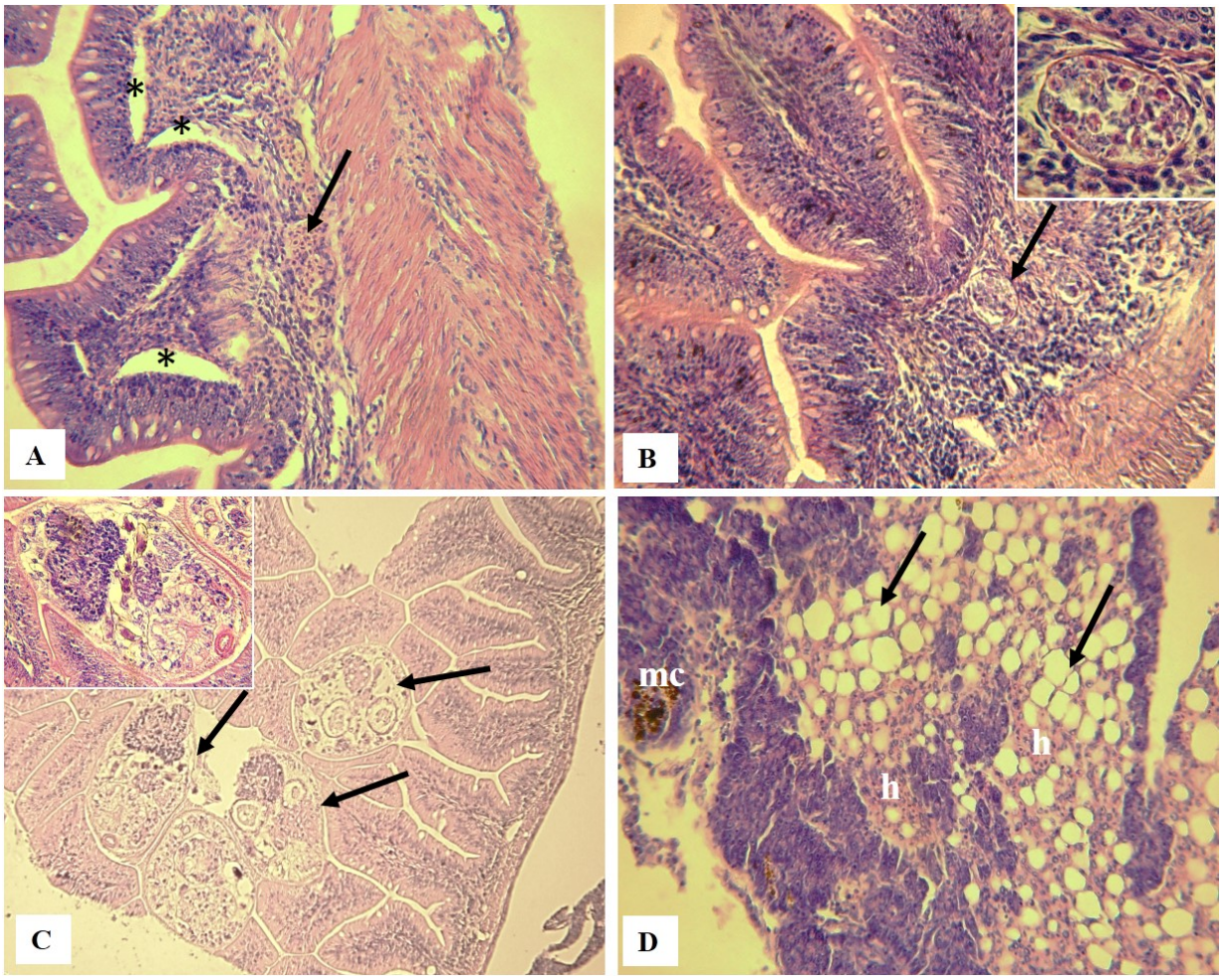

Fig. 3. Histopathological alterations in the gastrointestinal tract and pancreas of the golden grey mullet: A, local edema (*) and hyperemia (↑) of the submucosal layer of the pyloric stomach (×400); B, inflammatory reaction (infiltration) and protozoan cysts (↑) in the submucosal layer of the pyloric stomach (×400); C, trematodes (↑) in the lumen of the pyloric caeca (×100); D, steatosis (cell fatty degeneration), hyperemia (↑), and melanomacrophage center in the exocrine portion of the pancreas (×400, hematoxylin-eosin). Mc denotes melanomacrophage center; h, haemorrhagia

Invasion with a parasitic Protozoa, presumably *Ichthyophonus* sp., was recorded in the most vascularized organs of the golden grey mullet (the kidneys, liver, and spleen) and in the pancreas. Necrotic alterations, as well as granulomas, or fibrous capsules typical for ichthyophonosis were revealed (Fig. 4A). *Ichthyophonus* sp. "resting spores" were surrounded by elongated radially located epithelioid cells (Fig. 4B), or an accumulation of leukocytes and necrotic cells around the parasite was observed. MCs were identified as well (Fig. 4C). *Ichthyophonus* sp. spores with signs of degeneration were registered (Fig. 4D). When applying histochemical methods of staining according to Gram, Romanowsky–Giemsa, and Ziehl–Neelsen, no other pathogenic agents were detected in granulomas.

Summing up the importance factors of organ alterations in the mullets studied, the fish were divided into two groups. The group 1 (conditionally healthy) included individuals with the sum of histopathological alterations ranging 0 to 8 conventional units (n = 22 specimens). The group 2 included fish with the total sum 9 to 16 conventional units (n = 11 specimens). The importance (severity) factors for each alteration and the incidence of histopathological alterations in organs and tissues in fish of each group are given in Table 1.

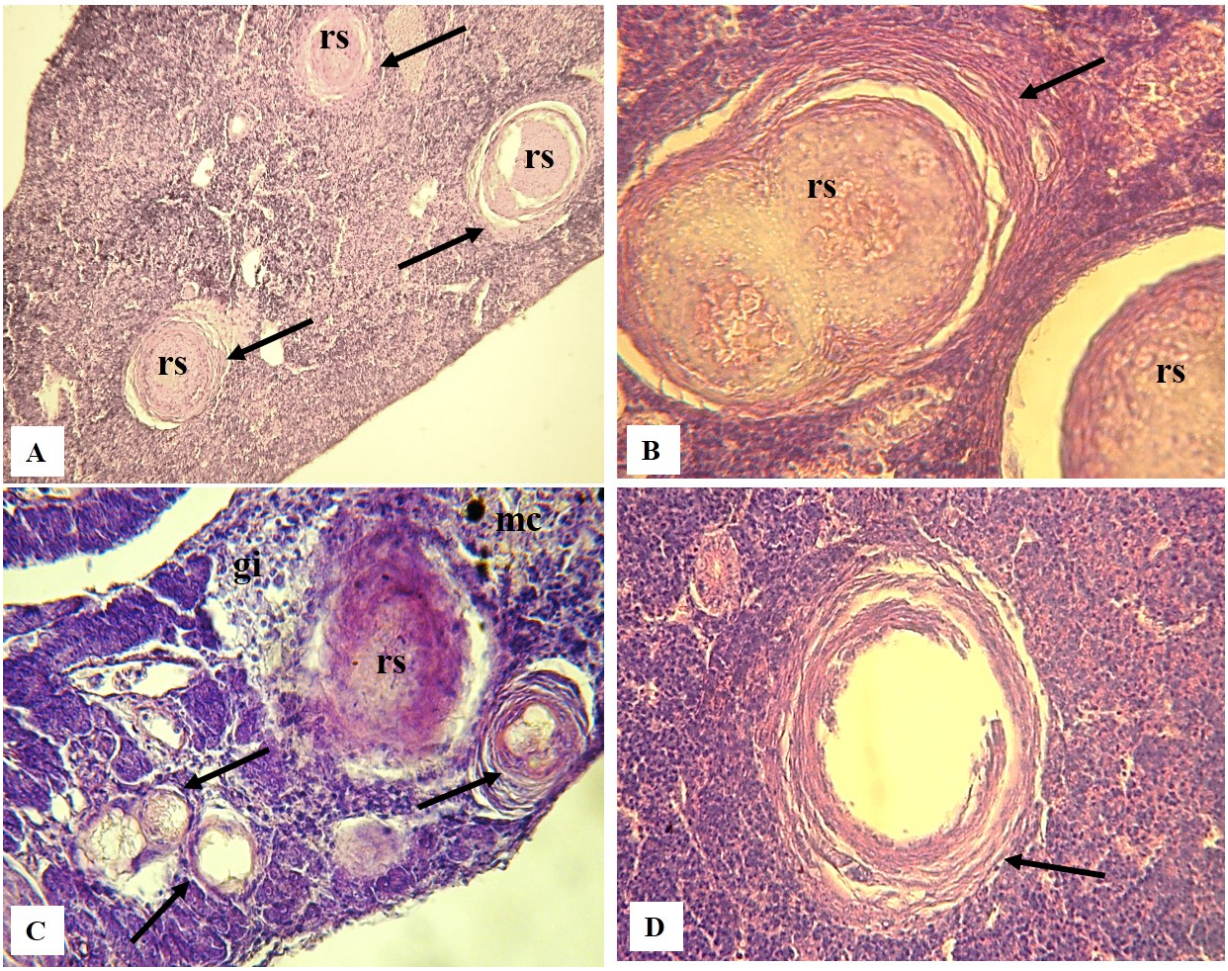

Fig. 4. Histopathological alterations in the golden grey mullet with ichthyophonosis: A, granulomas (↑) around "resting spores"; presumably, *Ichthyophonus* sp. in the hematopoietic tissue of the kidney (×100); B, epithelioid cells around the parasite (↑) (×1000, hematoxylin-eosin); C, melanomacrophage center and granulomatous inflammation around the parasite; spores with signs of degeneration are visible (↑) (×400, Romanowsky–Giemsa staining); D, empty "resting spore" in the kidney (↑) (×400, hematoxylin-eosin). Rs denotes "resting spore"; gi, granulomatous inflammation; mc, melanomacrophage center

Table 1. Incidence (%) of histopathological alterations in organs and tissues of the golden grey mullet (in each organ of one specimen, several different lesions could be detected). The importance (severity) factor for each alteration is indicated in brackets

Reaction pattern	Organ	Pathology	Incidence, %	
			Group 1	Group 2
Regressive alterations	Gills	Local necrosis of the respiratory epithelium cells of gill lamellae (3)	5.3	18.2
Progressive alterations		Hyperplasia of the respiratory epithelium of gill lamellae (2)	52.6	63.6
		Adhesion of gill lamellae (2)	5.3	9.1
Parasites		Single monogenean parasites on gill lamellae (2)	15.8	18.2
		Single *Trichodina* on gill lamellae (2)	21.1	18.2
		Protozoan cysts in gill filaments (2)	5.3	9.1

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Reaction pattern	Organ	Pathology	Incidence, %	
			Group 1	Group 2
Regressive alterations	Kidneys	Macrophage melanization around blood vessels (1)	5.3	9.1
		Melanomacrophage centers in the hematopoietic tissue of the kidney (1)	5.3	36.4
		Local vacuolization of the renal tubule epithelium (1)	0	18.2
		Hyaline droplet degeneration of nephrocytes (1)	0	45.5
		Renal tubule cells necrosis (2)	0	27.3
		Necrosis of individual renal tubules (3)	0	9.1
		Nephrocalcinosis (1)	10.6	45.5
Inflammation		Granulomas in the hematopoietic tissue of the kidney (2)	0	27.3
Parasites		Microorganisms (plasmodia of microsporidia or myxosporidia) in the lumen of the renal tubules (1)	5.3	18.2
		Ichthyophonus sp. (2)	0	27.3
Circulatory disorders	Liver	Dilation of blood vessels (1)	5.3	9.1
Regressive alterations		Local vacuolization of hepatocytes (1)	57.9	27.3
		Melanomacrophage centers (1)	21.1	27.3
		Fatty degeneration of hepatocytes (1)	10.6	36.4
		Local deposition of ceroid in hepatocytes (1)	15.8	18.2
		Nuclear pleomorphism of hepatocytes (2)	0	36.4
		Focal necrosis of hepatocytes (2)	5.3	27.3
Inflammation		Local inflammatory reaction around blood vessels / bile ducts (2)	57.9 / 36.8	81.8 / 18.2
		Granulomas (2)	5.3	27.3
Parasites		*Ichthyophonus* sp. (2)	5.3	27.3
Regressive alterations	Gastro-intestinal tract	Local mucosal cell necrosis (2)	5.3	9.1
Inflammation		Inflammatory reaction in the submucosal layer of the pyloric stomach and pyloric caeca (2)	31.6	63.6
Parasites		Nematodes in the lumen of the gastrointestinal tract (1)	5.3	9.1
		Trematodes in the lumen of the pyloric stomach and pyloric caeca (1)	15.8	36.4
		Microorganisms in the submucosal layer of the stomach (2)	15.8	45.5
Circulatory disorders	Pancreas	Hemorrhages in the exocrine tissue (1)	0	9.1
Regressive alterations		Melanomacrophage centers (1)	31.6	36.4
		Steatosis (2)	0	27.3
Inflammation		Granulomas in the exocrine portion (2)	5.3	54.5
Parasites		*Ichthyophonus* sp. (2)	5.3	54.5
Regressive alterations	Spleen	Melanomacrophage centers (1)	31.6	45.5
Inflammation		Local granulomas (2)	5.3	27.3
Parasites		*Ichthyophonus* sp. (2)	5.3	27.3

The reaction patterns of histological response varied significantly in the analyzed organs. The most frequent ones were regressive alterations and pathogenic agents (see Table 1). Inflammatory reactions were recorded in all the organs, except for the gills, in which progressive alterations were revealed (hyperplasia of the respiratory epithelium and adhesion of gill lamellae). Circulatory disorders were observed in the liver and pancreas only, although their incidence was insignificant (found in 5.3–9.1 % of fish). When assessing the prevalence of alterations in the golden grey mullet organs using the scoring system, severe lesions (these with scores 4 and 5) were not detected. In the fish of the group 1, most alterations of the importance factor 1 were revealed, with the incidence ranging 5.3 to 81.8 % (Table 1), whereas the distribution of lesion in organs did not exceed 20 % (score 1) (Table 2). The alterations of the importance factor 2 (the distribution of lesion 1–2) were recorded in the gills, liver, gastrointestinal tract, and pancreas (Table 2).

In the golden grey mullet of the group 2, alterations of the importance factors 1–3 were found, their incidence in organs accounted for 9.1–63.6 % and the distribution of lesion in the organ was 1–3. In these fish, like in the individuals of the group 1, the most frequently revealed alterations belonged to the importance factor 1, with the distribution of lesion equal to 1. The alterations characteristic of the importance factor 2 were predominantly focal (scores 1 and 2). Histopathological alterations of the importance factor 3 were recorded in the gills and kidneys only, with the incidence ranging 18.2 and 9.1 %, respectively; the distribution of lesion was 1 (see Table 2).

Table 2. Incidence (%) of histopathological alterations in organs and tissues of the golden grey mullet using the scoring system for the distribution of lesion. The importance (severity) factor for each alteration is indicated in brackets

Organ	Pathology	Incidence, % Group 1 / Group 2			
		0*	1	2	3
Gills	Local necrosis of the respiratory epithelium cells of gill lamellae (3)	94.7 / 81.8	5.3 / 18.2	0 / 0	0 / 0
	Hyperplasia of the respiratory epithelium of gill lamellae (2)	42.1 / 36.4	31.6 / 18.2	26.3 / 45.4	0 / 0
	Adhesion of gill lamellae (2)	94.7 / 90.9	5.3 / 9.1	0 / 0	0 / 0
	Single monogenean parasites on gill lamellae (2)	84.2 / 81.8	15.8 / 18.2	0 / 0	0 / 0
	Single *Trichodina* on gill lamellae (2)	78.9 / 81.8	21.1 / 18.2	0 / 0	0 / 0
	Protozoan cysts in gill filaments (2)	94.7 / 90.9	5.3 / 9.1	0 / 0	0 / 0
Kidneys	Macrophage melanization around blood vessels (1)	94.7 / 90.9	5.3 / 9.1	0 / 0	0 / 0
	Melanomacrophage centers in the hematopoietic tissue of the kidney (1)	94.7 / 63.6	5.3 / 36.4	0 / 0	0 / 0
	Local vacuolization of the renal tubule epithelium (1)	100 / 81.8	0 / 18.2	0 / 0	0 / 0
	Hyaline droplet degeneration of nephrocytes (1)	100 / 54.5	0 / 45.5	0 / 0	0 / 0
	Renal tubule cells necrosis (2)	100 / 81.7	0 / 18.2	0 / 9.1	0 / 0
	Necrosis of individual renal tubules (3)	100 / 90.9	0 / 9.1	0 / 0	0 / 0
	Nephrocalcinosis (1)	89.4 / 54.5	10.6 / 45.5	0 / 0	0 / 0
	Granulomas in the hematopoietic tissue of the kidney (2)	100 / 72.7	0 / 9.1	0 / 9.1	0 / 9.1
	Microorganisms (plasmodia of microsporidia or myxosporidia) in the lumen of the renal tubules (1)	94.7 / 81.8	5.3 / 18.2	0 / 0	0 / 0
	Ichthyophonus sp. (2)	100 / 72.7	0 / 9.1	0 / 18.2	0 / 0

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Organ	Pathology	Incidence, % Group 1 / Group 2			
		0*	1	2	3
Liver	Dilation of blood vessels (1)	94.7 / 90.9	5.3 / 9.1	0 / 0	0 / 0
	Local vacuolization of hepatocytes (1)	42.1 / 72.7	57.9 / 18.2	0 / 9.1	0 / 0
	Melanomacrophage centers (1)	78.9 / 72.7	21.1 / 27.3	0 / 0	0 / 0
	Fatty degeneration of hepatocytes (1)	89.4 / 63.6	10.6 / 27.3	0 / 9.1	0 / 0
	Local deposition of ceroid in hepatocytes (1)	84.2 / 81.8	10.5 / 9.1	5.3 / 9.1	0 / 0
	Nuclear pleomorphism of hepatocytes (2)	100 / 63.6	0 / 36.4	0 / 0	0 / 0
	Focal necrosis of hepatocytes (2)	94.7 / 72.7	5.3 / 27.3	0 / 0	0 / 0
	Local inflammatory reaction around blood vessels (2)	42.1 / 18.2	52.6 / 63.6	5.3 / 18.2	0 / 0
	Local inflammatory reaction around bile ducts (2)	63.2 / 81.8	36.8 / 18.2	0 / 0	0 / 0
	Granulomas (2)	94.7 / 72.7	0 / 9.1	0 / 9.1	5.3 / 9.1
	Ichthyophonus sp. (2)	97.4 / 72.7	0 / 18.2	0 / 0	5.3 / 9.1
Gastro-intestinal tract	Local mucosal cell necrosis (2)	94.7 / 90.9	5.3 / 9.1	0 / 0	0 / 0
	Inflammatory reaction in the submucosal layer of the pyloric stomach and pyloric caeca (2)	68.4 / 36.4	31.6 / 63.6	0 / 0	0 / 0
	Nematodes in the lumen of the gastrointestinal tract (1)	94.7 / 90.9	5.3 / 9.1	0 / 0	0 / 0
	Trematodes in the lumen of the pyloric stomach and pyloric caeca (1)	84.2 / 63.6	15.8 / 36.4	0 / 0	0 / 0
	Microorganisms in the submucosal layer of the stomach (2)	84.2 / 54.5	15.8 / 45.5	0 / 0	0 / 0
Pancreas	Hemorrhages in the exocrine tissue (1)	100 / 90.9	0 / 0	0 / 9.1	0 / 0
	Melanomacrophage centers (1)	68.4 / 63.6	26.3 / 27.3	5.3 / 9.1	0 / 0
	Steatosis (2)	100 / 72.7	0 / 9.1	0 / 18.2	0 / 0
	Granulomas in the exocrine portion (2)	94.7 / 45.5	5.3 / 36.3	0 / 9.1	0 / 9.1
	Ichthyophonus sp. (2)	94.7 / 45.5	5.3 / 36.3	0 / 9.1	0 / 9.1
Spleen	Melanomacrophage centers (1)	68.4 / 54.5	31.6 / 36.4	0 / 9.1	0 / 0
	Local granulomas (2)	94.7 / 72.7	5.3 / 9.1	0 / 9.1	0 / 9.1
	Ichthyophonus sp. (2)	94.7 / 72.7	5.3 / 9.1	0 / 9.1	0 / 9.1

Note: * denotes the distribution of lesion [0, absent or normal; 1, low (≤ 20 %); 2, moderate (21–40 %); 3, often (41–60 %)].

When carrying out a statistical analysis for two groups of the golden grey mullet, significant differences in the values of organ alteration indices were registered in the kidneys, liver, gastrointestinal tract, and pancreas (Table 3). The values of the total index of fish pathology were also significantly different (see Table 3).

Biochemical research. No significant differences between the level of oxidized proteins and TBARS in the liver of the mullets in the compared groups were recorded (Table 4).

There were no significant differences in the activity of aminotransferases between the groups 1 and 2. However, the activity tended to increase in the liver of fish with more pronounced histopathological alterations. As found, ALP activity was significantly higher, and urea content was lower in the liver of the conditionally healthy mullets (see Table 4).

At the same time, the content of total protein, albumin, and glucose in the tissues of infected and conditionally healthy fish did not differ significantly (Table 4).

Table 3. Values of organ alteration indices ($M \pm m$) for the golden grey mullet

Group	Organ alteration index						Total index of fish pathology, IT
	Gills, I_g	Kidneys, I_k	Liver, I_l	Gastrointestinal tract, I_{gt}	Pancreas, I_p	Spleen, I_s	
1	2.84 ± 3.00	0.84 ± 1.50	2.27 ± 2.21	1.16 ± 1.30	0.89 ± 1.24	0.47 ± 0.51	8.21 ± 5.63
2	4.36 ± 3.20	**4.63 ± 1.91****	**4.54 ± 2.69***	**3.00 ± 1.78****	**3.18 ± 2.08****	1.36 ± 1.80	**21.09 ± 6.09****

Note: indices are expressed in conventional units. In bold, the values for fish of the groups 1 and 2 are highlighted, with significant difference at $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

Table 4. Several biochemical parameters ($M \pm m$) in the liver and blood serum of the golden grey mullet

Parameter	Group 1 ($n = 22$)	Group 2 ($n = 11$)
Liver		
TBARS, nmol TBA·mg^{-1} protein	19.94 ± 2.77	18.02 ± 3.37
C_{356}, optical units·mg^{-1} protein	0.020 ± 0.002	0.024 ± 0.006
C_{370}, optical units·mg^{-1} protein	0.026 ± 0.004	0.027 ± 0.006
C_{430}, optical units·mg^{-1} protein	0.016 ± 0.003	0.019 ± 0.004
C_{530}, optical units·mg^{-1} protein	0.008 ± 0.001	0.008 ± 0.001
ALT, μmol·h^{-1}·mg^{-1} protein	0.091 ± 0.02	0.13 ± 0.02
AST, μmol·h^{-1}·mg^{-1} protein	0.21 ± 0.03	0.35 ± 0.07
ALP, nmol·sec^{-1}·mg^{-1} protein	677 ± 114	324 ± 60*
Urea, mmol·g^{-1} wet tissue	0.42 ± 0.036	1.07 ± 0.25*
Blood serum		
Total protein, mg·mL^{-1}	14.59 ± 1.49	14.05 ± 0.71
Albumin, mg·mL^{-1}	8.35 ± 1.55	8.91 ± 0.59
Glucose, mmol·L^{-1}	3.8 ± 0.76	2.69 ± 0.24

Note: * indicates significant differences between the values for fish of the groups 1 and 2, $p < 0.05$.

Thus, the results of the biochemical research in the tissues of the conditionally healthy mullets and individuals with more pronounced histopathological alterations allowed to establish certain peculiarities resulting from both the level of parasitic invasion and the severity and nature of histopathological alterations in fish organs.

DISCUSSION

The analysis of fish pathologies detected visually is an available method for assessing their health status. The visual signs of pathology registered by us in juvenile mullets were negligible. The pathogenic agents identified in the studied fish were represented by several species from different taxonomic groups: Protozoa, Monogenea, Trematoda, and Nematoda.

Comparative statistical analysis of histological alterations in organs of *C. auratus* from two examined groups revealed significant differences in the values of indices of kidney's alterations (Table 3). Substantially, it was caused by regressive alterations, with the greatest portion of MCs in the hematopoietic tissue, hyaline droplet degeneration of nephrocytes, and renal tubular nephrocalcinosis; all with the importance factor 1. Destructive alterations in the cells of the renal tubules (necrosis), with the importance factors 2 and 3, were detected in the group 2 alone (see Table 1).

In the liver, the histopathology pattern is not so unambiguous. Specifically, regressive alterations – fatty degeneration of hepatocytes, nuclear pleomorphism, and necrosis of hepatocytes – were recorded much more often in the infected golden grey mullets, while an inflammatory reaction – infiltration – around blood vessels and bile ducts was observed in fish from both groups (Table 1).

In the submucous layer of the pyloric stomach and pyloric caeca, the inflammatory reaction was recorded two times more often in the infected mullets, and parasite cysts were found three times more often. The incidence of trematodes in the lumen of the gastrointestinal tract was also two times higher in the group 2 (see Table 1). In the pancreas in the infected fish, steatosis was a distinctive feature of histopathological alterations.

Importantly, in the mullets of the group 2, *Ichthyophonus* sp. had a pathological effect on the liver, kidneys, spleen, and pancreas. The incidence of this pathogen was the highest in the pancreas (54.5 %) (Table 1). In conditionally healthy fish, *Ichthyophonus* sp. was found in the pancreas and spleen, but its incidence was significantly lower (5.3 %).

Thus, the most severe histopathological alterations detected in the golden grey mullet were caused by a parasitic protozoan, presumably *Ichthyophonus* sp. To date, *Ichthyophonus* sp. has been recorded in more than 100 species of cultivated and wild fish from seawater and freshwater of middle and tropical latitudes, and the list of its hosts keeps growing (Gavryuseva, 2007 ; Gaevskaya, 2004 ; Floyd-Rump et al., 2017 ; Noga, 2010 ; Osman et al., 2015). In mullets, the disease is recorded in the waters of Portugal, South Africa, and Japan, as well as in the North Atlantic (Gaevskaya, 2004 ; Ovcharenko, 2015). The disorders revealed in the tissues are typical for the chronic form of ichthyophonosis (Noga, 2010). As the disease progresses, an extensive granulomatous reaction leads to cirrhosis and atrophy of the affected organs that results in replacing most normal tissue by reticuloendothelial granulation tissue (Noga, 2010). Apparently, ichthyophonosis is a significant cause of chronic mortality in some populations of wild marine fish (Ovcharenko, 2015). As known, the severity of ichthyophonosis course is affected by water temperature and by fish species, sex, and age as well (Floyd-Rump et al., 2017 ; Osman et al., 2015).

Other pathogenic agents did not cause severe, irreversible histopathological alterations. Apparently, the inflammatory reaction in the submucosal layer of the gastrointestinal tract of fish resulted from the invasion by Protozoa, presumably myxosporidia. To verify the etiological agent of the inflammatory process in the gastrointestinal tract, further complex parasitological and histological studies are required. According to the literature data, 13 species of myxosporidia were identified in *C. auratus* in the Black Sea (Yurakhno, 2009). Among them, three species – *Myxobolus adeli* n. sp. (syn.: *M. improvisus* Isjumova, 1964), *M. exiguus*, and *M. muelleri* – invaded the gastrointestinal tract of fish.

In the golden grey mullet, we found no severe disorders caused by parasitic worms. Minor alterations recorded in the mucous layer were reversible. Nematodes were single, and their incidence was low (in 5.3–9.1 % of fish). Under natural conditions, trematodes in the lumen of the gastrointestinal tract do not cause significant damage (Gaevskaya, 2004 ; Dmitrieva & Gaevskaya, 2001).

No statistically significant histopathological alterations in the gills of the golden grey mullet were revealed since pathogenic agents (trichodines and monogeneans) were found in both groups of fish. Trichodines are widespread ectocommensals of the gills and skin of marine and freshwater hydrobionts. These parasites have strong pathogenic effect (excessive mucus secretion, destruction of gills,

anorexia, and respiratory failure) on fish fry and juveniles in mariculture (Gaevskaya, 2004 ; Noga, 2010). In our studies, we recorded single trichodines in the gills of the mullet and moderate hyperplasia of the respiratory epithelium of gill lamellae. Monogeneans caused more severe pathology – local necrosis and hyperplasia of epithelial cells of gill lamellae at the site of the parasite attachment. In some individuals, both trichodines and monogeneans were observed. Probably, synergistic effect of the mentioned ectoparasites can aggravate pathological processes in the gills.

The histopathological disorders with the importance factor 1 revealed in the mullets were reversible; the alterations with the importance factor 2 were local; and the disorders with the importance factor 3 were focal, *i. e.* only individual cells were damaged (see Table 2). According to the results of the histological studies, the health status of the most examined fish was satisfactory.

To assess the negative effect of parasitic invasions on the health status of fish, it is recommended to use LPO and PO parameters reflecting the level of tissue damage under oxidative stress. In particular, an increase in the level of LPO and PO was registered for the liver of sea trout *Salmo trutta* in case of ulcerative skin necrosis caused by the bacteria *Aeromonas hydrophila* (Kurhalyuk & Tkachenko, 2011). In the studies of the yellow perch *Perca flavescens* – both conditionally healthy fish (10 or less specimens) and those infected with metacercariae *Apophallus brevis* (> 10 individuals) – from reference and contaminated areas, the following was stated: the level of TBARS was higher in the liver of infected fish from both locations. The characteristics revealed were explained by the development of foci of chronic inflammation at the site of the parasite invasion in the fish muscles and skin (Marcogliese et al., 2005). In this work, the level of LPO and PO (Table 4) in the liver of the compared groups had no significant differences; so, there are no biochemical signs of cytolysis in the liver of the mullets, which is consistent with the data on pathomorphological analysis (see Table 1). The index of histopathological alterations in the liver was significantly higher in fish from the group 2, but most recorded histopathological alterations in the liver of the mullets from both groups had the importance factor 1, *i. e.* had no necrotic alterations related to the disruption of the cell integrity, and were reversible.

Another important biomarker recommended for assessing the functional state of the liver is aminotransferase enzymes. As a result of peramination catalyzed by aminotransferases, pyruvate, oxaloacetate, and α-ketoglutarate are formed, which are necessary for the synthesis of amino acids and serve as a substrate for gluconeogenesis. A compensatory increase in the activity of aminotransferases in fish liver was shown under the effect of various stress factors (Banaee et al., 2012, 2014). At the same time, chronic and/or rather strong effects can result in the disruption of the cell membrane integrity, "release" of aminotransferases into the blood, and decrease in their activity in fish liver (Kavitha et al., 2010 ; Kole et al., 2014). A rise in the activity of both aminotransferases was registered in the blood serum of the African sharptooth catfish *Clarias gariepinus* infected with *Trypanosoma mukasai* (Osman et al., 2009). In the studies of the Chinook salmon *Oncorhynchus tshawytscha*, both healthy and those with ichthyophonosis, no significant differences were found between the activity of ALT in the blood serum of the compared groups, while the activity of AST was significantly higher in the serum of healthy fish (Feist et al., 2015). Other authors established a rise in the activity of ALT and AST in the blood serum of fish with complex invasion compared to the activity in uninfected individuals (Nnabuchi et al., 2015 ; Noor et al., 2010). In our study, the activity of AST and ALT in the liver of the mullets (Table 4), as well as the level of LPO and PO (see Table 4), did not differ significantly in the compared groups. This indicates the lack of oxidative damage to hepatocytes and is consistent with the data on pathomorphological analysis (Table 1).

The content of urea, the end product of protein metabolism, was higher in the liver of the fish from the group 2 (Table 4), which may result from the kidney and gill excretory dysfunction (Table 3). An increase in the index of histopathological alterations was registered for the kidneys and gills of the mullet from the group 2 with the significant differences for the first case. Local vacuolization and necrosis of renal tubule cells, as well as necrosis of individual renal tubules, were recorded in 18.2, 27.3, and 9.1 % of fish from the group 2, respectively, whereas in the mullets from the group 1, these histopathological alterations were not observed (Table 1). The ratio of individuals with histopathological alterations in the gills (adhesion of gill lamellae, local cell necrosis, and hyperplasia of the respiratory epithelium of gill lamellae) was also higher in the group 2 than in the group 1 (Table 1). In the Nile tilapia *Oreochromis niloticus*, an increase in serum urea content was registered in individuals infected with a protozoan *Trichodina* sp. and monogenean *Cichlidogyrus* sp. (Noor et al., 2010). The studies of *Clarias gariepinus* and *C. anguilaris*, both healthy and infected with parasites, showed a rise in urea content in the blood serum of infected individuals. The authors attributed this to the gill damage by the protozoan *Trichodina acuta* (Nnabuchi et al., 2015).

The activity of ALP was significantly lower in the liver of the mullets from the group 2 (Table 4). The lack of shifts in the level of LPO and PO, as well as necrotic alterations in the liver of the compared groups, excludes cytolysis of hepatocytes in the fish from group 2. At the same time, the ratio of incidence of all the histological alterations was higher in the liver of the mullets from the group 2, except for signs of the local inflammatory reaction around the bile ducts, the incidence of which was higher in the liver of the fish from the group 1 (Table 1). Apparently, the revealed characteristics are the reason for an increase in ALP activity, a marker of cholestasis, in the liver of fish from the group 1. It requires further study of histopathological alterations of the gallbladder and its ducts under parasitic invasions. In the gallbladder of mullets from Sevastopol water area and in the Black Sea, 17 species of myxosporidia were recorded (Yurakhno, 2009 ; Yurakhno & Ovcharenko, 2014). A rise in the activity of ALP in the blood serum of clariids was observed under complex invasion. The authors explained it by blockage of the bile ducts by parasites (Nnabuchi et al., 2015). Other researchers did not record any significant differences between the activity of ALP in the blood serum of healthy *O. tshawytscha* and individuals with ichthyophonosis (Feist et al., 2015).

Comparative analysis of the parameters of protein metabolism (total protein and albumin) and carbohydrate metabolism (glucose content) in the blood serum of the mullets from two groups showed no significant differences (see Table 4). It indicates a satisfactory health status and the reversibility of the most of the identified histological alterations as well.

Conclusion. In the organs of the golden grey mullet, the histopathological alterations referring to four types of the reaction patterns were detected: circulatory disorders, regressive and progressive alterations, and inflammatory processes. Furthermore, parasites were identified. Most of the recorded alterations belonged to the importance factor 1 (these were reversible). Such pathologies are typical for weak toxic process, which could be initiated by both biotic factors (pathogenic agents) and abiotic ones (in particular, anthropogenic load).

The modified scoring system of histopathological alterations and a semi-quantitative analysis of the alterations revealed in juvenile mullets allowed to transform the data on qualitative tissue damage into quantitative parameters and to obtain evidences about the health status of the fish studied.

Pathogenic agents found in juvenile mullets were represented by several species from different taxonomic groups: Protozoa, Monogenea, Trematoda, and Nematoda. The most severe histopathological alterations were caused by a parasitic protozoan, presumably *Ichthyophonus* sp. In other mullets studied, structural damages registered in organs and tissues were reversible, and pathogenic agents did not cause severe histopathological alterations.

Significant differences in the values of organ alteration indices were recorded in the kidneys, liver, gastrointestinal tract, and pancreas of the fish studied. These were reversible (in most cases, the nuclei and cell membranes were not destroyed), and this is confirmed by the data of the biochemical studies.

In conditionally healthy and infected mullets, the level of lipid peroxidation and protein oxidation and the activity of aminotransferases in the liver did not differ significantly, which also indicates the lack of oxidative damage of hepatocytes. An increase in urea content in the liver of *C. auratus* from the group 2 might result from the kidney and gill excretory dysfunction (it was confirmed histologically). The concentration of total protein, albumin, and glucose in the blood serum of the mullets from the compared groups did not differ significantly, which also is a sign of a satisfactory health status of the fish and the reversibility of most of the identified histological alterations.

The results obtained confirm high informativeness of applying the semi-quantitative analysis of histopathological alterations and the set of biochemical parameters for assessing the health status of the golden grey mullet.

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ПАТОМОРФОЛОГИЧЕСКИЕ И БИОХИМИЧЕСКИЕ ИССЛЕДОВАНИЯ КЕФАЛИ СИНГИЛЯ *CHELON AURATUS* (RISSO, 1810) В АКВАТОРИИ ЮГО-ЗАПАДНОГО КРЫМА (ЧЁРНОЕ МОРЕ)

Т. В. Гаврюсева, Т. Б. Сигачева, И. И. Чеснокова

ФГБУН ФИЦ «Институт биологии южных морей имени А. О. Ковалевского РАН»,
Севастополь, Российская Федерация
E-mail: *gavrt2004@mail.ru*

Кефаль сингиль *Chelon auratus* (Risso, 1810) (Mugilidae) является ценным промысловым видом, занимающим первое место по объёмам вылова среди аборигенных черноморских кефалевых рыб в коммерческом и любительском рыболовстве. Высокая значимость сингиля в региональном промысле демерсальных рыб требует разработки системы оценки состояния здоровья этого вида. Проведение подобного рода исследований включает комплексное применение

биохимических и патоморфологических методов, что позволяет изучить изменения, происходящие в организме рыб, до появления видимых проявлений, нарушения процессов роста и размножения, снижения промысловых размеров и сокращения численности популяции. Целью работы было исследовать патоморфологические изменения в сочетании с некоторыми биохимическими показателями тканей кефали сингиля для оценки состояния здоровья рыб. Были проведены визуальный осмотр и патологоанатомическое вскрытие рыб. Для гистологического анализа пробы жабр, печени, почек, желудочно-кишечного тракта, селезёнки и поджелудочной железы были зафиксированы в растворе Дэвидсона и обработаны с использованием стандартных методов. Состояние организма *C. auratus* на основе гистологических исследований определяли с применением модифицированного полуколичественного анализа альтераций по методике Берне с соавторами и оценки распространённости повреждений в органах согласно балльной системе. Выяснили факторы значимости выявленных повреждений, значения индексов альтерации органов и общий индекс патологии кефалей. При проведении биохимических исследований определяли содержание продуктов окислительной модификации белков, перекисного окисления липидов и мочевины, активность аминотрансфераз и щелочной фосфатазы в печени, концентрацию альбумина и глюкозы в сыворотке крови. В органах кефали сингиля обнаружены гистопатологические изменения четырёх типов (нарушение кровообращения, регрессивные и прогрессивные изменения, воспалительные процессы), а также паразиты. Паразитарные агенты, выявленные у молоди кефали, представлены несколькими видами разных систематических групп (простейшие, моногенеи, трематоды, нематоды). Наиболее тяжёлые гистопатологические изменения были вызваны паразитарным простейшим, предположительно *Ichthyophonus* sp. При полуколичественной оценке обнаруженных альтераций рыб условно разделили на две группы — условно здоровых и заражённых особей; между ними провели сравнительный анализ патоморфологических данных и некоторых биохимических показателей. Выявлены достоверные различия в значениях индексов альтераций органов между двумя группами *C. auratus* в почках, печени, желудочно-кишечном тракте и поджелудочной железе. Значения общего индекса патологии рыб также достоверно отличались. При биохимических исследованиях определено достоверное увеличение содержания мочевины в печени рыб из 2-й группы, которое может свидетельствовать о нарушении экскреторной функции почек и жабр (подтверждено гистологически). Значимых отличий содержания продуктов перекисного окисления липидов и окислительной модификации белков, а также активности аминотрансфераз в печени условно здоровых и заражённых рыб не выявлено. Результаты работы подтверждают высокую информативность исследованных показателей для оценки состояния здоровья кефали сингиля.

Ключевые слова: кефаль сингиль, гистопатологические изменения, биохимические показатели, полуколичественный анализ, Чёрное море