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THE EFFECTS OF HEAVY METALS ON THE INTESTINE OF CULTURED *ACIPENSER STELLATUS* (PALLAS, 1771)

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Abstract: The intestine of the fish, together with the gills, is an important organ in the uptake of heavy metals. Using histopathology, this study aimed to investigate the effects of two heavy metals (copper and zinc) on the intestine of *Acipenser stellatus* juveniles. Intestinal samples from stellate sturgeon were fixed in 10% formalin for 24h. The specimens were processed through paraffin embedding and 7 micrometer sections were cut, stained by Hematoxylin and Eosin and observed under light microscope. Exposure to heavy metals had significant effects on the histological organization of the intestine. The results of the present study showed that heavy metals exert their toxic effects on different intestinal layers, most of the lesions being observed on the intestinal mucosa. Of the two metals, copper induced major histopathological changes in the intestinal mucosa, compared to zinc.

Keywords: *Acipenser stellatus*, copper, zinc, intestine, histology

1. INTRODUCTION

The Danube sturgeon species are some of the most globally important species of fish. Romania, Bulgaria, and a small part of Serbia hold the only current viable wild sturgeon populations in the European Union.

Currently, of the six sturgeon species that existed in the Danube, it is considered that only five species are present: *Huso huso, Acipenser gueldenstaedtii, A. nudiventris, A. ruthenus, A. stellatus. Acipenser sturio* is considered extinct in the Danube (Williot et al., 2002), while *Acipenser nudiventris* is still present but facing extinction in the next years (Jaric et al., 2009).

Acipenser stellatus is one of the sturgeon species found in the Black Sea, from where it migrates for reproduction in the Danube River (Radu et al., 2008).

Water pollution is seriously impacting the sturgeon's populations in the Danube River, alongside with overfishing and poaching, gravel extraction, destruction of critical habitats by construction of dams, and poaching (Lenhardt et al., 2008).

The pollution is high with copper and zinc in the Danube River, compared to other metals, as shown by the ICPDR Report (2015).

In fish, both a branchial and intestinal uptake of metals (Bury et al., 2003), is taking place.

Since just a few studies have tried to investigate the effects of copper and zinc on fish intestine, especially in sturgeons, in this study we aimed to highlight the histopathological changes induced by the two metals, on the intestine of *Acipenser stellatus* juveniles.

The sturgeon intestine consists of three portions: the anterior intestine, the spiral valve, and the terminal portion of the intestine or rectum (Singer & Ballantyne, 2005).

The three regions of the intestine are specialized in different degrees for the processes of digestion and absorption (Gawlicka et al., 1995).

Since the anterior intestine is considered to be the portion of the digestive tract with the highest absorption capacity for copper and zinc, respectively for their binding to mucus (Ojo & Wood, 2007), we

mainly investigated the changes induced by these heavy metals at this segment.

2. MATERIALS AND METHODS

2.1. Experimental exposure

Acipenser stellatus juveniles were obtained from Brateş Research Hatchery, Galati, and transferred to the Institute of Research and Development for Aquatic Ecology, Fishing and Aquaculture, Galati, Romania. The juveniles with a length of 26.9 ± 1.68 cm and a weight of 40.8 ± 6.05 g were obtained by artificial reproduction, from wild parents caught in the Danube River. After the transfer, the juveniles were left to acclimate to laboratory conditions for 7 days (Vasile et al., 2019).

Physical and chemical parameters of the water were measured daily. Dechlorinated tap water with a pH value of 8.26 ± 0.12, a temperature of 16.1 ± 0.31°C and dissolved oxygen with a value of 8.81 ± 0.43 $mg.l^{-1}$ was used in the experiments. The total hardness -titration method (Popa et al., 2001) was 340 $mg.l^{-1}$ $CaCO_3$ (Ca: 80.16 $mg.l^{-1}$; Mg: 34.03 $mg.l^{-1}$). The water was renewed daily (50% of the water), uneaten feed, faeces and organic matter being siphoned after feeding (Vasile et al., 2019).

Throughout the experiment the juveniles were fed with 2% of body weight with granulated food. The amount of food offered is the amount recommended by OECD Guide 204 and considered optimal for sturgeon growth (Mohseni et al., 2006). We have tried to avoid additional histopathological changes in the digestive tract induced by starvation (Gisbert & Doroshov, 2003). Feeding was stopped 24 hours before sacrifice.

Acipenser stellatus juveniles (10 individuals per each group) were exposed for 7 and 14 days to two different concentration of copper and zinc. The concentrations used in the present study were 10% and 25% of the lethal concentration (LC50-96h) that Vasile et al., (2015) previously determined as 0.547 $mg.l^{-1}$ for Cu^{2+}, and 34.225 $mg.l^{-1}$ Zn^{2+}. The control group was kept in tap water only.

2.2 Chemical substances

Copper sulfate pentahydrate (CAS 7758-99-8; purity 99.09%) and zinc sulfate heptahydrate (CAS 7446-20-0, purity 99.67%) were obtained from Chemical Company Iasi (Romania).

2.3 Histological analysis

The juveniles were captured and anesthetized by immersion to sedation in 2-phenoxyethanol solution. They were sacrificed by evisceration, the digestive tube being immediately taken and fixed in 10% formalin for histopathological determinations.

The tissues were processed using the standard histological technique: dehydration in an ethanol series, embedding in paraffin, and serially sectioning at 7 m. Sections were stained with hematoxylin and eosin (H/E). Microphotographs were taken with an Olympus microscope and camera.

All experimental procedures and animal maintenance were in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes.

3. RESULTS AND DISCUSSIONS

3.1. Normal histological structure of the intestine

In control group, the histological analysis of the intestinal wall showed a similar organization as to that of other fishes, respectively the mucosa with epithelium composed of enterocytes and mucosal cells, the *lamina propria* of conjunctive nature. Also visible were the submucosa, the muscularis externa made up of two layers of smooth muscle cells, and on the outside the serosa.

The enterocytes are ordered in a single layer, showing the margin as a brush and the nuclei located in the basal portion (Fig. 1). The muscularis externa has a homogeneous appearance (Fig. 2).

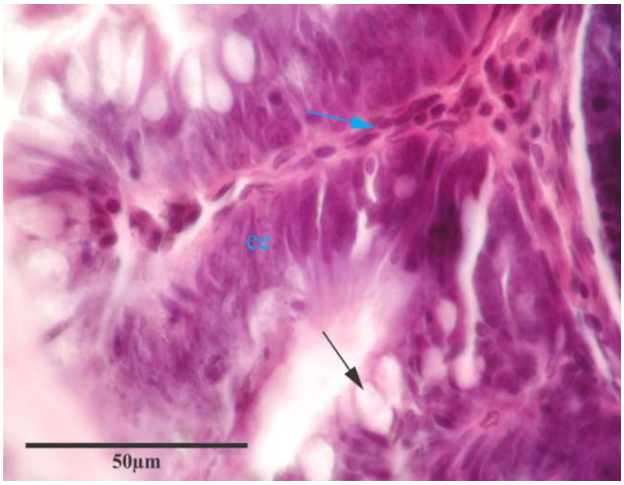


Figure 1. Intestine of *Acipenser stellatus*, H&E staining: mucous cells (black arrow), enterocytes in intestinal epithelium (ee), *lamina propria* (blue arrow), x40 (original photo).

The anterior portion of the intestine is the place of the digestive processes under the action of its own enzymes and those secreted by the accessory glands. Also, in this region, it takes place the uptake of

nutrients. The anterior intestine also plays an endocrine role, producing hormones such as secretin that stimulate pancreatic secretion (Morrison, 2007).

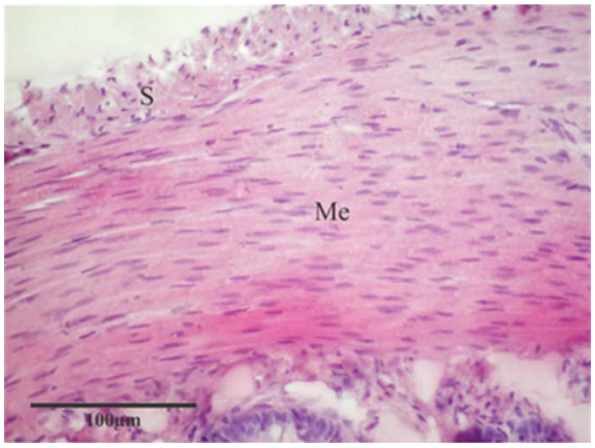


Figure 2. Intestine of *Acipenser stellatus*, H&E staining: homogenous muscularis externa (Me) and serosa (S), x40 (original photo).

3.2. Histopathological changes induced by copper

The reduced concentration of copper (0.054 $mg.l^{-1}$) after 7 days of exposure induced significant changes in the intestine of exposed individuals.

Exposure to 0.054 $mg.l^{-1}$ copper for 7 days and 14 days induced changes in intestinal epithelium - hypertrophy and the appearance of intercellular spaces (Fig. 3), in the *lamina propria* - retraction of connective tissue, capillary hyperemia, oedema and infiltration of inflammatory cells in the submucosa and muscularis externa (Fig. 4, Fig. 5, Fig. 6).

In individuals intoxicated with 0.136 $mg.l^{-1}$ copper for 7 days, histopathological changes were evident in the intestine, including hypertrophy of epithelial layer (Fig. 7), apical dilation and fusion of the villi (Fig. 8). Inflammatory cells infiltrating into the epithelial layer and the underlying connective tissue can also be observed. In both individuals exposed for 7 days and those exposed for 14 days, the high dose of copper led to the appearance of necrotic lesions at the level of the intestinal villi (fig.9, fig.10).

3.3. Histopathological changes induced by zinc

Individuals intoxicated with 3.42 $mg.l^{-1}$ zinc for 7 days frequently showed capillary hyperemia in the intestinal villi, associated with the infiltration of inflammatory cells (Fig. 11, Fig. 12, Fig. 13). Also, moderate vacuolization of the enterocyte cytoplasm was observed (Fig. 14).

Fish intoxicated with 8.55 $mg.l^{-1}$ zinc for 7 and 14 days showed pronounced changes in the intestinal epithelium. There was also a more intense vacuolization of the cytoplasm of enterocytes, and the glandular cells had large mucous vacuoles (Fig. 16, Fig. 18). We observed as well the appearance of intercellular spaces (Fig. 15), hypertrophy of the nuclei (Fig. 15, Fig. 18). At the level of the muscularis externa, infiltration of the inflammatory cells took place, which had an atypical appearance, with wide corridors between the muscle fibers (Fig. 17). The intestine is one of the target organs of xenobiotics in both food and water, these toxic compounds directly affecting the regulation of water and ion balance given the important function of osmoregulation of this organ.

The intestinal epithelium is very sensitive to the action of toxic agents because it frequently comes in direct contact with them, not only through food, water but also through the content of bile secretion.

In the present histopathological study, we indicated the presence of some changes in the intestinal level, after exposure to copper and zinc.

Given that the administration of heavy metals was accomplished through water, and not by food, the changes observed in the intestine, it is most likely due to the presence of heavy metals in the intestine reached through bile excretion, which contributes to the elimination of xenobiotic compounds. Considering the fact that the water drinking rate is very low, in fresh water, there is no question of accumulation of metals on this route.

Previous studies have shown that fish are capable of bioconcentrating a wide variety of toxic substances including heavy metals and excreting them through the bile at the intestinal level.

Considering that the stellate sturgeons consumed the food as soon this was administered, there is no question of contamination of the food with the metals in the water. The remaining granules were collected as soon as the individuals stopped feeding.

In general, the distribution of lesions in the mucosa of the digestive tract in intoxicated individuals was uneven, with an alternation between regions with normal appearance and areas deeply affected by the toxic agent.

Both metals, caused changes in the intestinal mucosa, after only 7 days of exposure. In the case of copper exposure, an increase in the lesion's severity was observed as the metal concentrations increases. As the concentration increases, from 0.054 $mg.l^{-1}$ to 0.136 $mg.l^{-1}$ after 7 days of exposure, an increase in the histopathological changes of the mucosal defense is observed.

Copper induced significant changes especially in the intestinal mucosa. From these changes we can mention, hypertrophy of the epithelium and mucous cells, dilatation of the intercellular space, the fusion

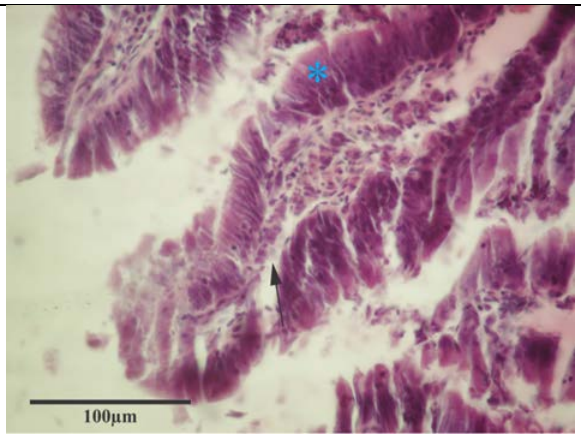


Figure 3. Intestine of *Acipenser stellatus* intoxicated with 0.054 mg.l^{-1} Cu^{2+}, 7 days, H&E staining: retraction of connective tissue (arrrow), hypertrophy of intestinal epithelium (*), x40 (original photo).

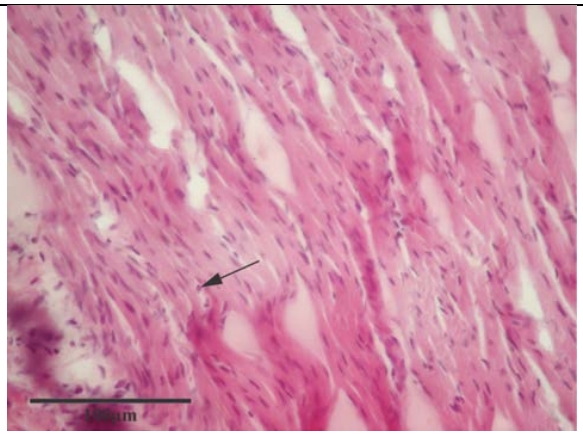


Figure 4. Intestine of *Acipenser stellatus* intoxicated with 0.054 mg.l^{-1} Cu^{2+}, 7 days, H&E staining: changes in the muscularis externa – oedema and infiltration of inflamatory cells, x40 (original photo).

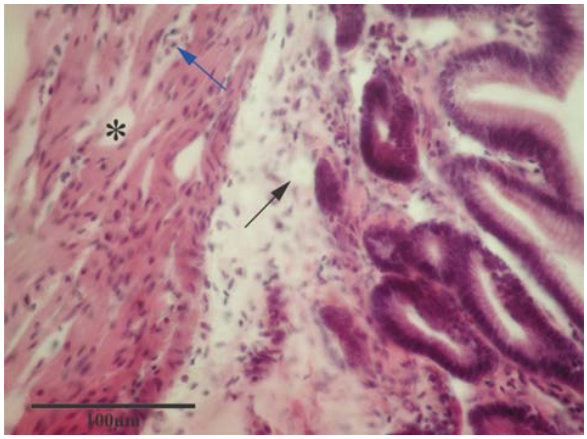


Figure 5. Intestine of *Acipenser stellatus* intoxicated with 0.054 mg.l^{-1} Cu^{2+}, 7 days, H&E staining: loss of homogenity and large spaces between fibres in muscularis externa (*), oedema between mucosa and submucosa (black arrow), inflamatory cells in muscularis externa (blue arrow), x40 (original photo).

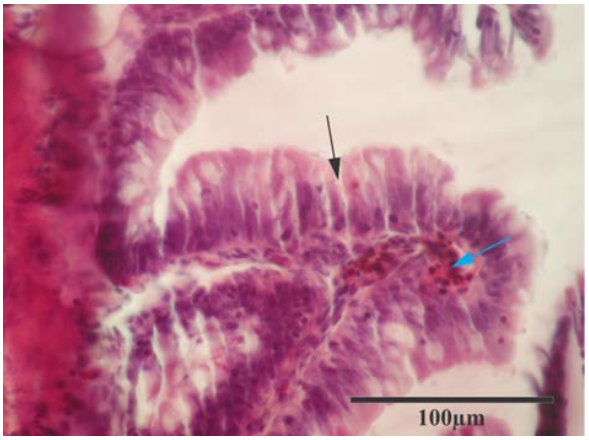


Figure 6. Intestine of *Acipenser stellatus* intoxicated with 0.054 mg.l^{-1} Cu^{2+}, 7 days, H&E staining: intercellular spaces in the epithelium (black arrow), inflamatory cells and hyperemia of capillary vessel (blue arrow), x40 (original photo).

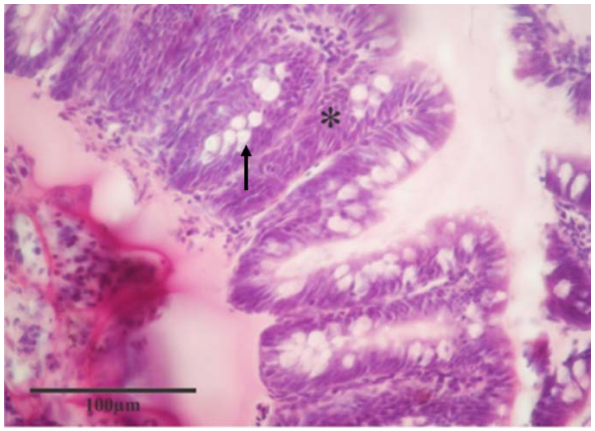


Figure 7. Intestine of *Acipenser stellatus* intoxicated with 0.136 mg.l^{-1} Cu^{2+}, 7 days, H&E staining: hipertrophy of epithelial layer (*), hypertrophy of mucous cells (arrow), x40 (original photo).

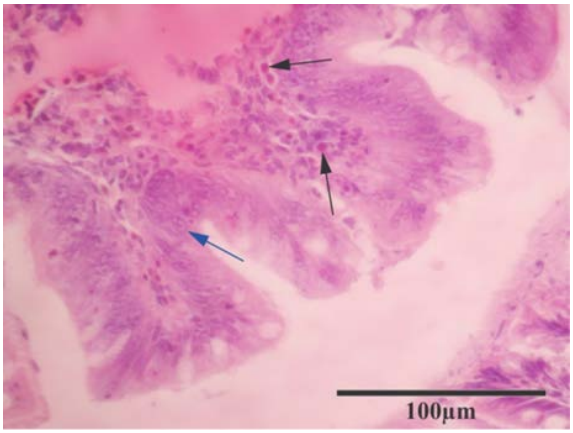


Figure 8. Intestine of *Acipenser stellatus* intoxicated with 0.136 mg.l^{-1} Cu^{2+}, 7 days, H&E staining: dilatation of intestinal villi in the apical region, epithelial cells with hypertrophied nuclei (blue arrow), inflamatory cells in epithelial layer and connective layer (black arrow), x40 (original photo).

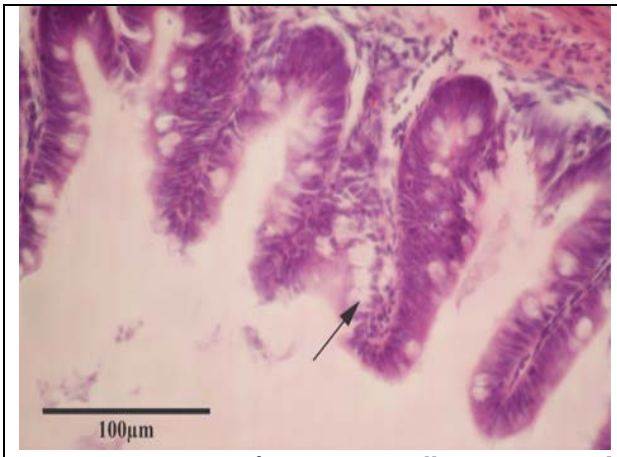


Figure 9. Intestine of *Acipenser stellatus* intoxicated with 0.136 mg.l^{-1} Cu^{2+}, 7days, H&E staining: necrosis (black arrow), x40 (original photo).

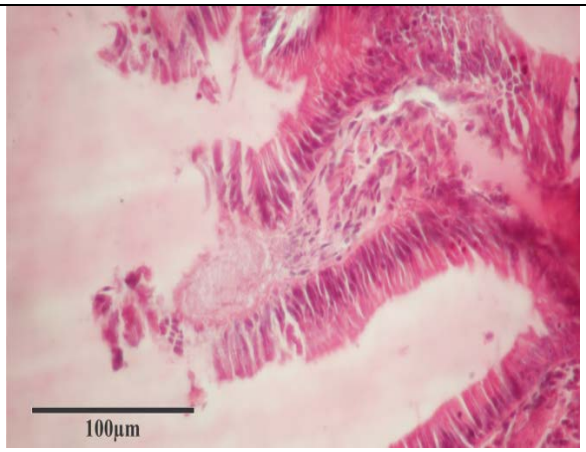


Figure 10. Intestine of *Acipenser stellatus* intoxicated with 0.136 mg.l^{-1} Cu^{2+}, 14 days, H&E staining: villi necrosis in apical region, x40 (original photo).

Figure 11. Intestine of *Acipenser stellatus* intoxicated with 3.42 mg.l^{-1} Zn^{2+}, 7 days, H&E staining: dilatation of intestinal villi, oedema between mucosa and submucosa (*), inflammatory cells and hyperemia of capillary vessel (arrow), x40 (original photo).

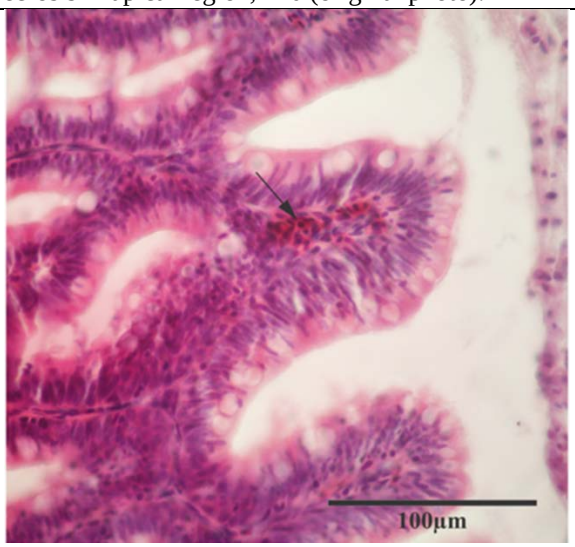


Figure 12. Intestine of *Acipenser stellatus* intoxicated with 3.42 mg.l^{-1} Zn^{2+}, 7 days, H&E staining: inflammatory cells and hyperemia of capillary vessel (arrow), x40 (original photo).

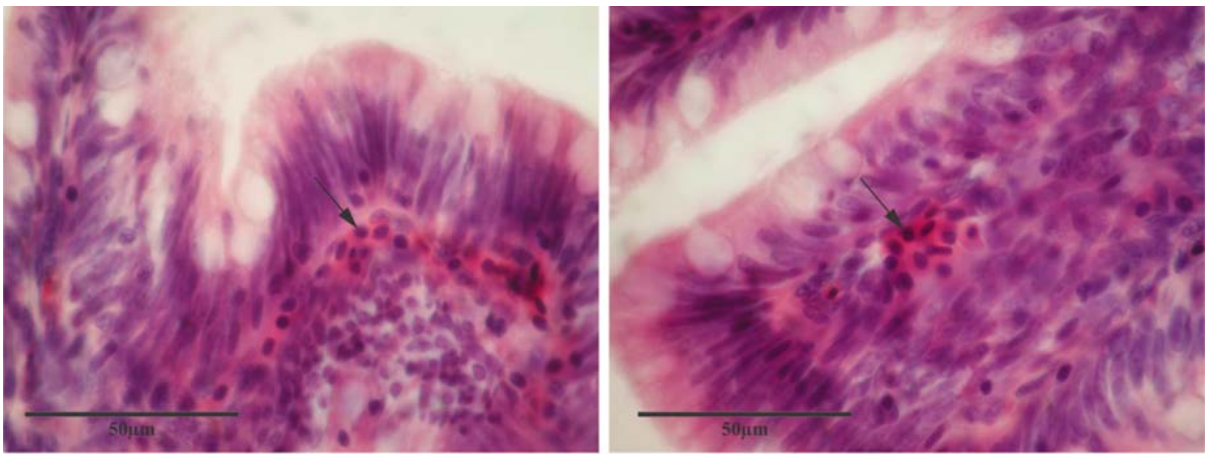


Figure 13. Intestine of *Acipenser stellatus* intoxicated with 3.42 mg.l^{-1} Zn^{2+}, 7 days, H&E staining: inflammatory cells in *lamina propria* and hyperemia of capillary vessel (arrow), x40 (original photo).

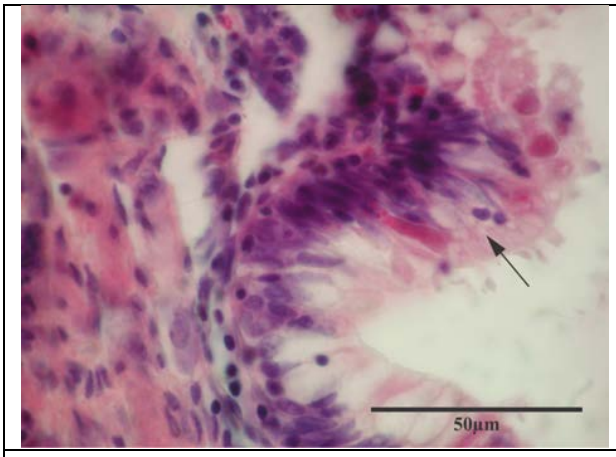
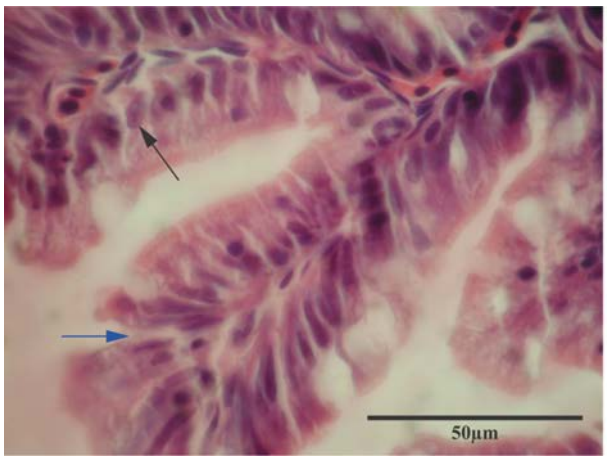


Figure14. Intestine of *Acipenser stellatus* intoxicated with 3.42 mg.l^{-1} Zn^{2+}, 7 days, H&E stainning: mucous vacuoles (arrow), x100 (original photo).



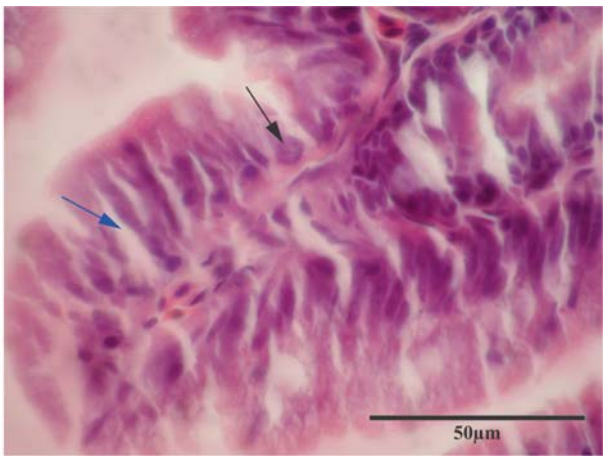


Figure15. Intestine of *Acipenser stellatus* intoxicated with 8.55 mg.l^{-1} Zn^{2+}, 14 days, H&E staining: nuclear hypertrophy (black arrow), intercellular spaces (blue arrow), x100 (original photo).

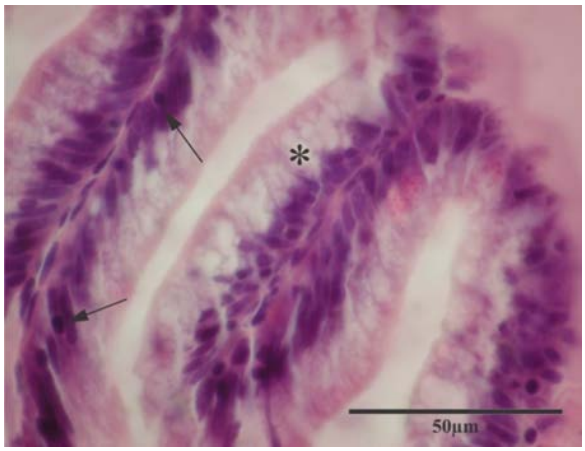


Figure16. Intestine of *Acipenser stellatus* intoxicated with 8.55 mg.l^{-1} Zn^{2+}, 14 days, H&E staining: vacuolization of epithelial cells cytoplasm (*), lymphocyte infiltration in epithelial layer (black arrow), x100 (original photo).

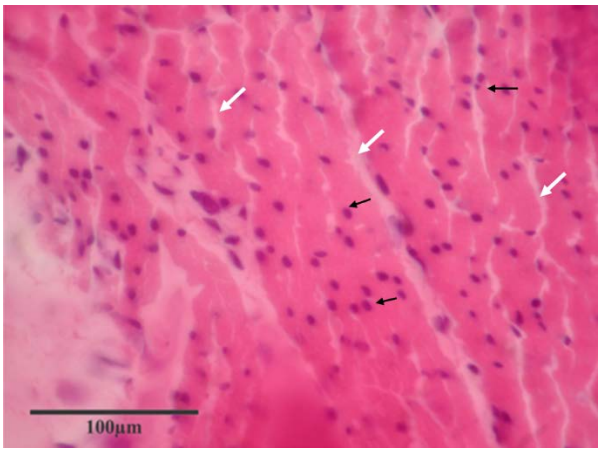


Figure17. Intestine of *Acipenser stellatus* intoxicated with 8.55 mg.l^{-1} Zn^{2+}, 14 days, H&E staining: inflammatory cells in muscularis externa (black arrow), corridors between muscular fibres (white arrow), x100 (original photo).

and dilatation of intestinal villi, increase of enterocytes stratification, the retraction of the connective tissue.

Also in the case of copper exposure, at the concentration of 0.136 mg.l^{-1}, 7 days of exposure, the appearance of necrosis was observed, as a result of the direct effect of the metal on the intestinal epithelium. Necrosis of epithelial tissue often results in an increase in the permeability of the epithelium for water and ions (Wendelaar & Lock, 2008).

Unlike copper, zinc exposure did not cause major histopathological changes in the mucosa. As a

defense reaction, at the concentration of 3.42 mg.l^{-1} starting with 7 days of exposure, a dilatation of the intestinal villi but also the vacuolization of the mucous cells, the infiltration of the inflammatory cells, and hyperemia were seen.

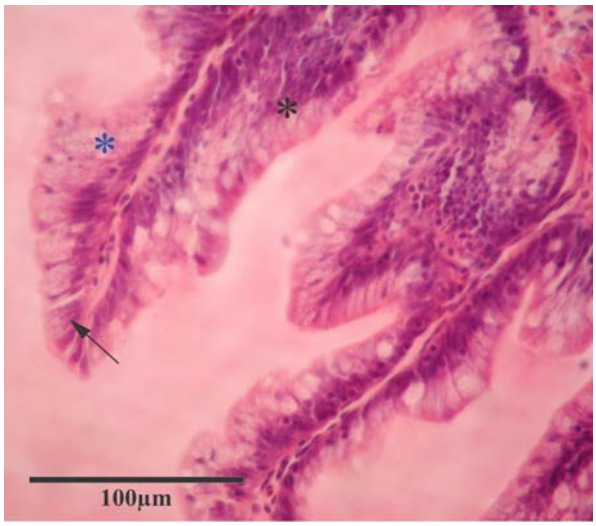


Figure 18. Intestine of *Acipenser stellatus* intoxicated with 8.55 mg.l^{-1} Zn^{2+}, 14 days, H&E staining: vacuolization of epithelial cells cytoplasm (*), nuclear hypertrophy (black arrow), epithelial hypertrophy (*), glandular cells with big mucous vacuoles (blue arrow), x40 (original photo).

Regarding the severity of the lesions observed in individuals exposed to heavy metal contamination, necrosis is one of the marked pathological changes. This lesion is generally irreversible, which can affect the normal functioning of the intestine.

The others lesions observed in the intestine of exposed stellate sturgeons, are most often reversible lesions, with the cessation of the action of the toxic agent, thus considered defense mechanisms. Since most of the intestinal changes were recorded at the mucosal level, this indicates that the bioaccumulation of metals occurs in the intestinal mucosa. The experiment of Handy et al., (2000) demonstrated that the highest concentration of copper accumulated in the intestine is localized to the mucosal cells.

Thus, the changes observed in the case of zinc exposure, within the present experiment, may indicate that in the intestine, the concentration of zinc was not high enough to induce significant changes in the intestinal mucosa.

Exposure to zinc (1.6 mg.l^{-1}) by water, did not result in significant accumulation of metal in the intestine of individuals of *Oncorhynchus mykiss* as observed by Kock & Bucher (1997).

This underlines the fact that mucus plays an important role in binding and precipitating the heavy metals, at the level of the epithelium, thus preventing the taking over of metals.

The transport of metals through the digestive tract involves a series of steps. The first step is to bind the metal to the mucus of the intestinal lumen, which can facilitate or reduce the retrieval of metals. The second stage involves the transfer of the metals from the lumen into the mucosal epithelium via the apical membranes of the enterocytes, the third stage being the export of the metals through their basolateral membrane to the blood or extracellular fluid (Ojo & Wood, 2007).

Pathological reactions at the level of the intestinal submucosa were represented by oedema between the mucosa and submucosa, especially at the lower concentrations of the two metals (10% LC50), after 7 days of exposure. Their presence indicates that both metals are capable of inducing an increase in vascular permeability, as well as disturbance of hydrostatic or osmotic pressure, thus causing fluid accumulation in the interstitial space. The presence of oedema is considered by Hanna et al., (2005) as a result of heavy metal absorption.

Hyperemia, next to oedema, represents a change in circulation that has occurred in individuals exposed to both metals. Increased blood flow to the *lamina propria* was due to dilation of arterioles, or an inflammatory process (Bernet et al., 1999).

The muscularis externa of the gastrointestinal tract may also undergo pathological changes. Among the changes induced in the muscularis externa, mainly oedema and the presence of inflammatory cells (lymphocytes) were observed, but also the loss of homogeneity and the appearance of large spaces between fibers in the muscularis externa.

The presence of lymphocytes in the muscularis externa indicates a chronic inflammation. The inflammatory processes induce a series of nonspecific transformations, easily confused with the effects of other types of physiological reactions, but they are additionally accompanied by the following characteristic signs: infiltration of tissues with leukocytes, the presence of an exudate rich in plasma proteins and the proliferation of the mononuclear phagocytic system. The inflammatory response is usually secondary to other types of lesions and is triggered by signals emitted by cells affected by necrosis.

The changes observed in the intestine are common lesions, observed in the case of exposure to heavy metals. Exposure to copper often induces hyperemia, degenerative changes (hypertrophy, vacuolization) of the intestinal epithelium, loss of structural integrity of the intestinal folds, necrosis (Maharajan et al., 2016).

Heavy metals are recognized to affect the intestinal bacterial communities (Mickeniene &

Syvokiene, 2001, 2008; Mickeniene et al., 2007), the diversity and abundance of bacteria in the digestive tract decreasing significantly. This, in conjunction with the changes induced by metals in the intestinal epithelium, indicates that normal digestive processes of absorption of nutrients, etc. are affected.

In bony fish, a series of changes in osmoregulation structures allow for continuous ionic and osmotic homeostasis. The intestine, although less studied, constitutes with the gills and kidneys, an important organ in the process of osmoregulation in sturgeons. Studies have shown that sturgeons drink water during acclimatization to seawater (Rodriguez et al., 2002; Zydlewski & Wilkie, 2013), in order prevent dehydration. But for its absorption in the intestine, the water must be desalinated in the esophagus by elimination of Na^+ and Cl^- ions, leading to reduced water osmolarity.

Water thus reaches the anterior intestine, which is the main region of the digestive tract for water absorption, due to the high concentration of Cl- ions (Allen et al., 2009).

Thus, the histopathological changes observed in the small intestine as a result of exposure to heavy metals may affect the processes of osmoregulation at the intestinal level that juvenile specimens entering the seawater must perform.

The study of Poleksic et al., (2010), on Danube sterlet individuals caught in the Lower Danube showed high concentrations of copper, zinc and iron at the intestinal level, compared to other metals, but the authors do not mention the region of the intestine used to determine the concentration of heavy metals. The intestine was the tissue with the second highest concentration, after the liver, of copper, zinc and nickel, followed by the gills and muscles with the lowest concentrations.

Also, the authors do not include in the study the histopathological changes induced in the intestinal level by the presence of these metals. Therefore, further studies on the influence of heavy metal toxicity on different intestinal segments are required.

4. CONCLUSIONS

In our experiment, exposure of stellate sturgeon (*Acipenser stellatus*) juveniles to heavy metals induced a series of histological changes in the intestine. Our study showed that heavy metals exert their toxic effects on different intestinal layers.

Both metals, in the individual experiments caused changes in the intestinal mucosa, after 7 and 14 days of exposure.

Most lesions observed in the intestinal mucosa, constitute defense mechanisms against the toxicity of heavy metals.

In the case of copper exposed individuals, a relationship was observed between the metal concentration and the severity of intestinal lesions. In contrast to copper, zinc exposure did not cause severe histopathological changes.

The major intestinal changes were recorded at the mucosal level, this indicating that the bioaccumulation of metals occurred at this level.

Since heavy metals can affect the intestinal bacterial communities, along with induced changes in the intestinal epithelium, it suggests that sturgeons may have problems with normal digestive processes of nutrient uptake. At the same time, given the role of the intestine in the osmoregulation process, heavy metals can influence this process through the observed histopathological changes.

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