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Histopathological Changes of the Countercurrent System in Fish Gills Constantly Exposed to Heavy Metal-Polluted Waters

Cambios Histopatológicos del Sistema de Contracorriente en Branquias de Peces Constantemente Expuestas en Aguas Contaminadas con Metales Pesados

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SUMMARY: Fish gills represent one of the most vulnerable target organs to aquatic pollutants. Extensive research shows that chronic exposure to heavy metals leads to structural lesions and impaired ion regulation. However, up to the moment, it is unclear how such disturbances affect the countercurrent exchange mechanism, which is essential in fish respiration. The present study aimed to provide a thorough description of the histological and functional responses of gills to heavy metal exposure, explaining how such modifications affect the countercurrent exchange. Paired gill samples were harvested from adult European chub *Squalius cephalus* (Linnaeus, 1758) collected from the Arie? River catchment (geographical area of Rosia Montana), a high-risk aquatic ecosystem where fauna is continuously exposed to toxic concentrations of metals. 10 % buffered formalin fixed gill samples were further demineralized, processed with the usual paraffin embedding technique and stained with the Goldner's trichrome method. The results showed a plethora of lesions, which were classified according to their magnitude into four different classes: adaptive, degenerative, inflammatory, and irreversible. The first three categories were present in almost all individuals, while the prevalence of the irreversible lesions was slightly lower. In such conditions, the countercurrent exchange mechanism is impaired even by initial modifications such as the lifting of the simple squamous covering epithelium. Due to a bigger distance between the lamellar axis and the exterior of the lamella, the oxygenated blood may find it harder to reach the destination point. Accordingly, the loss of lamellar structural integrity reduces the gradient necessary for optimal gas exchange, both protective adaptations and detrimental outcomes collectively undermining the physiological countercurrent exchange mechanism. These findings demonstrate that metal-induced histopathological damage might undermine countercurrent gas exchange, providing a reliable biomarker framework for assessing aquatic heavy metal toxicity.

KEY WORDS: Aquatic ecotoxicology; Gill morphology; Physiological mechanism; Respiratory efficiency; Sentinel species.

INTRODUCTION

Gills are vital multifunctional organs crucial for maintaining homeostasis in aquatic environments. Their main role is the oxygenation of blood, yet their physiological significance extends far beyond respiration (Mokhtar, 2021). Gills are also critically involved in acid–base balance, osmoregulation, ion exchange, and the excretion of nitrogenous wastes—primarily in the form of ammonia (Genten *et al*., 2009). These combined functions make the gills a highly dynamic and metabolically active interface between the fish and its aquatic environment. Structurally, the gill epithelium is extremely thin and characterized by a remarkably large surface area, a configuration that facilitates rapid and efficient gas diffusion. However, this structural delicacy exposes the organ to a high degree of vulnerability, rendering it susceptible to physical, chemical, and biological stressors present in the surrounding water. Even minor alterations to epithelial integrity can result in significant respiratory and osmoregulatory disturbances (Mumford *et al*., 2007; Genten *et al*., 2009; Mokhtar, 2021).

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Anatomically, the gills are composed of arches from which the primary lamellae, or filaments, project outward in a comb-like display. The surface of each primary lamella is further expanded by numerous secondary lamellae, arranged as regular semilunar folds along the dorsal and ventral surfaces. This hierarchical lamellar system provides an enormous surface area that is essential for efficient gas exchange and the removal of metabolic waste products. The secondary lamellae serve as the primary sites of gaseous exchange and are composed of a delicate, simple epithelium of squamous pavement cells (PVC). These cells are supported and separated by contractile pillar cells, which maintain the structural rigidity of the lamellae and regulate the diameter of lacunar spaces through which blood flows. The spacing of pillar cells—approximately 9–10 mm apart—ensures optimal diffusion distances between blood and water while preserving lamellar integrity (Genten *et al*., 2009; Mokhtar, 2021).

A fundamental feature underpinning the exceptional respiratory efficiency of the gills is the countercurrent exchange mechanism. In this system, water flows over the lamellae in the opposite direction to the flow of blood within the capillaries, from afferent to efferent arterioles. This arrangement maintains a continuous diffusion gradient, allowing for the progressive transfer of oxygen from water into the blood and for carbon dioxide to diffuse in the opposite direction. Through this process, oxygen extraction efficiencies ranging from 50 % to 80 % are achieved (Genten *et al*., 2009). Following oxygenation, blood exits the secondary lamellae through the efferent lamellar arteries, converging into the dorsal aorta for systemic distribution. The ventral aorta, in turn, delivers deoxygenated blood from the heart to the gill arches via the afferent branchial arteries, ensuring a closed and efficient circulation. Collectively, the microanatomical organization of the gills and the precision of the countercurrent exchange mechanism exemplify an evolutionary optimization of respiratory and osmoregulatory efficiency in aquatic vertebrates (Olson, 1991; Mumford *et al*., 2007; Genten *et al*., 2009).

Given that the gills account for more than half of the total surface area of a fish (Playle, 1998; Yancheva *et al*., 2016), it is not surprising that they represent one of the major target organs for waterborne toxicants. Acting as the primary interface between the internal environment of the fish and the surrounding water, gills are the main route through which toxicants penetrate into the organism (Carpene & Vasak 1989; Perry & Laurent, 1993; Pourang, 1995; Tkacheva *et al*., 2004; Rosseland *et al*., 2007; Yancheva *et al*., 2016). Consequently, they are among the most sensitive structures for detecting the physiological and histopathological effects of aquatic pollution. The gill surface possesses metal-binding ligands, and bioaccumulation often results from electrostatic interactions between positively charged metal ions and negatively charged binding sites on the gill epithelium (Playle *et al*., 1998; Yancheva *et al*., 2016).

The Rosia Montana–Abrud sector of the Aries River catchment in western Romania is recognized as one of the most historically and environmentally impacted mining areas in Europe. Mining and ore processing for gold, silver, copper, lead, zinc, and other non-ferrous metals have been ongoing in this region for over two millennia (Banaduc *et al*., 2021). Numerous studies have reported that both historical and current mining operations have induced long-term, synergistic negative effects on aquatic ecosystems, resulting in a chronic state of environmental pollution (Bird *et al*., 2005; Levei *et al*., 2011; Banaduc *et al*., 2021). The region's rivers receive mine effluents and acid drainage waters characterized by high concentrations of suspended solids, sulfates, chlorides, and heavy metals such as Fe, Cu, Pb, Zn, Cd, Mn, and others, often accompanied by highly acidic pH values sometimes below 3 (Dumitrel *et al*., 2015; Banaduc *et al*., 2021). Concentrations of iron and copper ions have been reported to exceed legal limits several times, while significant excesses were also observed for manganese and cadmium (Dumitrel *et al*., 2015). Consequently, water quality assessments classify the Aries River downstream of the Rosia Montana and Rosia Poieni mining zones as class V—bad ecological status, indicating severe degradation (Levei *et al*., 2011; Dumitrel *et al*., 2015). Recent research confirms that mining waste discharge, abandoned tailings, and uncontrolled seepage remain major sources of contamination (Glevitzky *et al*., 2025). The persistent accumulation of metals in sediments has led to bioaccumulation in aquatic organisms, including fish and benthic invertebrates, which exhibit pronounced alterations as a result of chronic exposure to heavy metals (Banaduc *et al*., 2021).

Thus, the Aries River catchment represents a high-risk ecosystem where aquatic fauna are continuously exposed to toxic concentrations of metals, making it an essential case study for evaluating pollutant-induced histopathological changes in fish gills. Therefore, the study aimed to understand the histological and functional responses of gills to metal exposure. Moreover, the mechanisms by which heavy metals alter countercurrent efficiency and respiratory physiology are fundamental for assessing aquatic toxicity.

MATERIAL AND METHOD

Sampling site

In order to select the proper aquatic environment for our purpose, after a rigorous study of the literature, the geographical area of Rosia Montana was selected. To facilitate field assessment, a decision tree was created (Fig. 1).

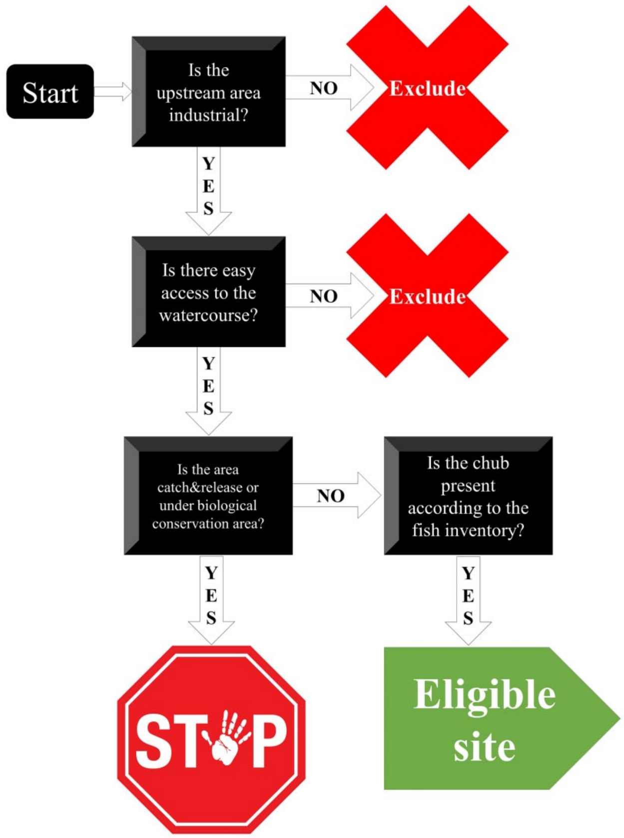

Fig. 1. Easy-to-use on the field decisional tree for a watershed polluted by industrial heavy metals.

In short, the aim was to identify aquatic sample sites (running waters from Rosia Montana geographical area, tributaries to the Aries River catchment) that are known to be polluted with heavy metals, where access to the water is easy and the withholding of the catches is allowed in the legal fishing season (no "catch & release" zone).

Biological material

The European chub *Squalius cephalus* (Linnaeus, 1758) demonstrated its capacity to represent a veritable sentinel species (Hájková *et al*., 2007; Hávelková *et al*., 2008; Krywult *et al*., 2008). Accordingly, in the present study a total number of 15 European chub were caught between July and September 2025 using the spinning fishing method, with artificial lures. Only adult individuals, above 25 cm (minimal legal length), were retained and immediately euthanized with an overdose of Eugenia cariophilata extract in water. Within the shortest time after euthanasia, the fish were anatomically dissected, and the paired gills were harvested. All the samples were instantly immersed in 10 % buffered formalin.

Histological assessment

Histological processing of the samples was performed in the histology laboratory of the Faculty of Veterinary Medicine at the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania. The samples were fixed for 5 days. For better processing, the following step involved demineralization with trichloroacetic acid 5 % for 7 days. Subsequently, the gills were processed with the usual paraffin embedding technique and sectioned at 5 µm thickness, using a rotary microtome (Leica Rotary microtome, Germany). The mounted sections were stained with Goldner's trichrome staining method (Suvarna *et al*., 2018). The microscopical assessment was carried out with an Olympus BX41 microscope equipped with an Olympus SC180 digital camera (Olympus, Japan), while the used software was Olympus CellSens.

Histopathological diagnosis: Microscopic images were independently evaluated by two pathologists, and lesions were classified accordingly.

RESULTS

The histological assessment revealed several morphological alterations in all the analyzed fish gill specimens. Moreover, the pathological findings are provoked by different mechanisms that permit classification into different severity categories, such as: adaptive or defense lesions, degenerative/necrotic or reversible lesions, inflammatory lesions, and irreversible or severe pathological lesions (Table I, Fig. 2).

Except for inflammatory lesions (Fig. 2 A, B, E), the other categories represent different steps of the severity of morphological changes, which is directly related to the exposure period length and intensity. Accordingly, the same specimen may develop all four categories of lesions, but in a different time frame.

The adaptive or defense lesions may be present after a slightly short period of exposure to a range of pollutants (Fig. 2C). Punctual changes like epithelial lifting/detachment (due to interstitial edema) associated with subepithelial clefts formation, or a mild epithelial hyperplasia, followed by secondary lamellae fusion were seen in all the analyzed specimens. At the same time, an increased mucus secretion was noticed, mostly on the apical region of the primary gill lamellae. Together with those cell-linked alterations, mild vascular lesions like vasodilatation were observed with a high prevalence.

The degenerative lesions were also observed with a high prevalence, but not in all the individuals. As stated before, they may represent a more advanced stage of the adaptive lesions that may be provoked by a longer exposure to the polluted environmental conditions. A frequent alteration was represented by the rupture of the epithelium. However, morphological changes at the level of the pillar cells were also frequently noticed (Fig. 2D).

Another major histological change identified in all analyzed slides was represented by the inflammatory lesions. In most cases, a prominent inflammatory infiltrate was observed in the apical region of the primary gill lamellae (Fig. 2E).

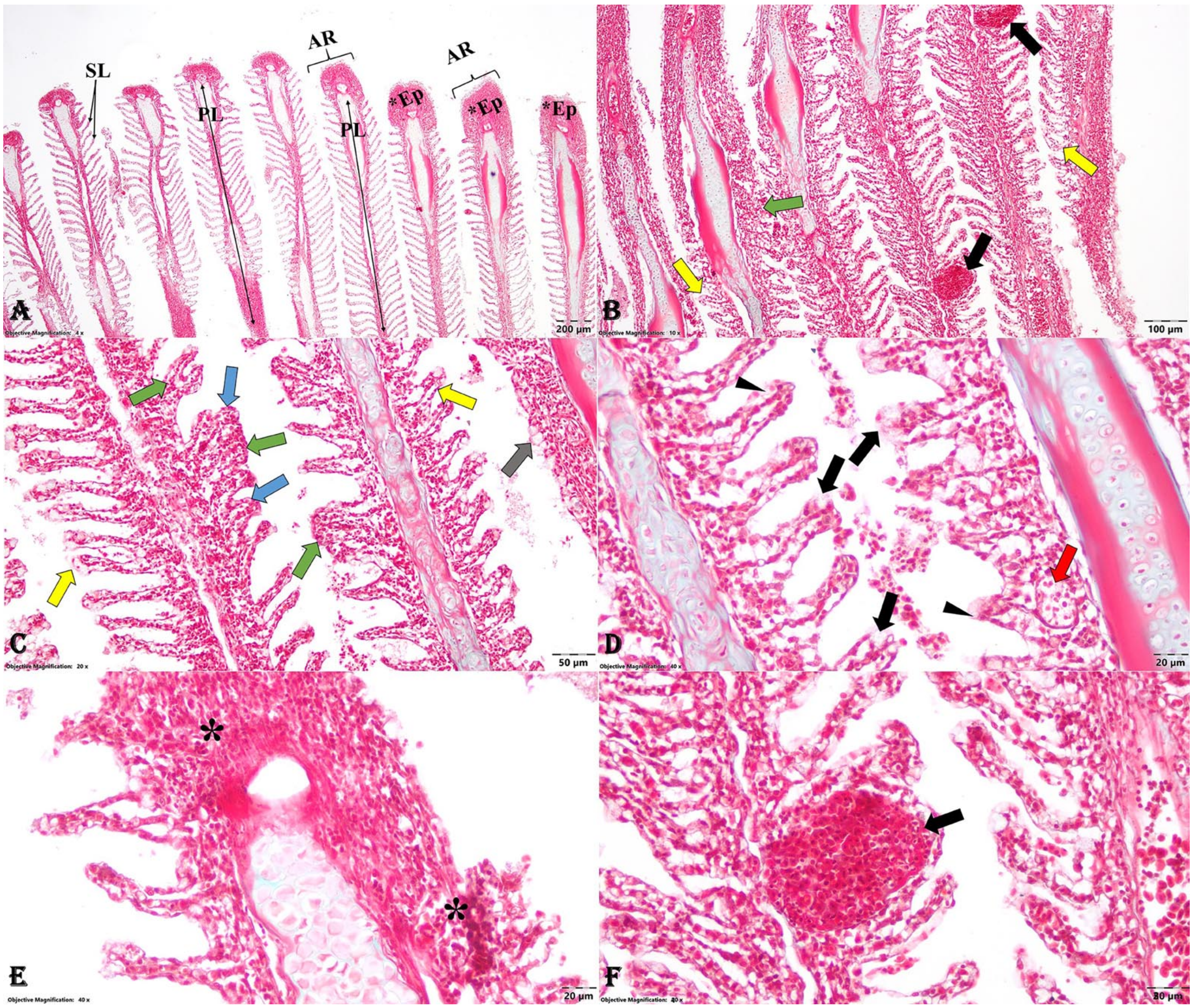

Fig. 2. Gill lesions found in fish that lived in environmental conditions polluted with heavy metals (Goldner's trichrome stain). A – 4X, Overview of affected gill lamellae presenting primary gills (doubled arrow PG) from which the secondary lamellae detach (SL); the apical region (AR) of the primary lamellae presents epithelial hyperplasia, with an increased number of mucous cells (*Ep). B – 10X, Lesions overview: epithelial detachment with associated subepithelial clefts (yellow arrows); multifocal areas with merging aspect of the secondary lamellae (green arrow); failure of the pillar cells to maintain the normal blood flow on the secondary lamellae, with the aneurysm formation as a consequence (black arrows). C – 20 X, A gill region where the adaptive or defense lesions predominate: interstitial edema, including epithelial lifting/detachment (yellow arrows), hyperplasia of epithelial cells (blue arrows), lamellar fusion (green arrows), mucus hypersecretion (grey arrow). D – 40X, Degenerative and necrotic lesions represented by: early epithelial degeneration denoted by cytoplasmic vacuolization (arrowheads) and pillar cell structural changes with the loss of mechanical support (red arrow); epithelial necrosis (black arrows). E – 40X, Inflammatory lesion mainly represented by the presence of a prominent lymphoid infiltrate in the gill structure (asterisks). F – 40X, Irreversible lesion displaying focal aneurysmal blood vessel dilation due to pillar cell failure (black arrow).

Regarding the most severe category of lesions, namely the irreversible injuries, they were occasionally detected in all assessed specimens. However, in several individuals, isolated failures of the pillar cells were noticed, with the appearance of the aneurysms as a consequence (Fig. 2F). At the same time, advanced epithelial degeneration with tissue loss, together with cellular necrosis was identified as well (Fig. 2E, F).

From the countercurrent point of view, even the primary or adaptive lesions, such as interstitial edema, may negatively interfere with the highly specific countercurrent exchange mechanism. A higher distance in between the blood and the surrounding water will increase the demanded time for oxygen passage throughout the multi-layered gill's barrier. Accordingly, even easily reversible gill lesions will impair the oxygenation rate of the fish.

Table I. Classification of gill lesions found in the analysed specimens.

No.	Lesion category	Severity	Type of lesion
1.	**Adaptive or defense**	Easily reversible It occurs as a protective reaction	• Interstitial edema (including epithelial lifting/detachment, subepithelial clefts) • Hyperplasia of epithelial cells • Lamellar fusion • Mucous hypersecretion and thickened mucous layer • Vasodilation of lamellar axis (early, adaptive stage)
2.	**Degenerative/ Necrotic**	The damage may be (hardly) reversed, but only if the exposure stops	• Early epithelial degeneration (cytoplasmic vacuolization), epithelial atrophy • Pillar cell structural changes (loss of mechanical support after vasodilation) • Scattered epithelial desquamation (necrosis of the branchial epithelium)
3.	**Inflammatory**	Represent immune response to pollutants Involve immune cell infiltration	• Infiltration of **lymphocytes and macrophages** • **Hyperplasia of chloride and mucous cells** associated with inflammation • **Possible fibrosis**
4.	**Irreversible**	Advanced, severe lesions that compromise gill function permanently even if the exposure stops	• Lamellar aneurysms (blood channel dilation due to pillar cell failure) • Extensive necrosis of gill epithelial cells • Advanced epithelial degeneration with tissue loss

DISCUSSION

The Rosia Montana–Abrud area within the Aries Basin is consistently classified as a high-risk (class V) zone for heavy metal contamination, with iron, copper, manganese, lead, and cadmium frequently exceeding permissible concentrations by several orders of magnitude (Levei *et al*., 2011; Dumitrel *et al*., 2015; Banaduc *et al*., 2021). The chronic exposure of aquatic biota to such metal-rich, acidic waters has been shown to induce significant physiological and histological disturbances in fish, including gill lesions and osmoregulatory dysfunctions, confirming that the region's long-standing mining legacy continues to pose severe ecological threats (Banaduc *et al*., 2021).

Most of the reported findings in the present study are supported by the scientific literature. Numerous studies have documented various histopathological alterations in fish gills exposed to contaminated water, both under experimental and natural conditions (Doughtie & Rao, 1983; Laurén & McDonald, 1985; Alazemi *et al*., 1996; Mazon *et al*., 2002; Rao *et al*., 2003, 2005; Camargo & Martinez, 2007; Matos *et al*., 2007; Velcheva *et al*., 2010). As it was presented in the results section, these changes can be either adaptive or destructive in nature (Tkacheva *et al*., 2004; Yancheva *et al*., 2016), reflecting the fish's attempt to mitigate damage while maintaining respiratory function. However, up to now, it is not clear how such histological changes influence the countercurrent mechanism.

The complex structural organization of the gills is central to their physiological performance, particularly in sustaining the efficiency of countercurrent gas exchange. The primary and secondary lamellae not only maximize surface area but also establish microenvironments where blood and water can flow in close proximity, separated by only a thin epithelial barrier (Genten *et al*., 2009). The thinness of the gill epithelium, coupled with the extensive vascularization of the lamellar network, is essential for maintaining a minimal diffusion distance, thereby facilitating the rapid transfer of respiratory gases (Olson, 1991). The pillar cells, positioned in regular arrays, play a dual role: they provide mechanical support against water pressure and actively regulate blood flow by modulating the width of the lacunar spaces. Through contraction or relaxation, these cells can influence perfusion rates and, consequently, the efficiency of oxygen uptake under varying environmental or metabolic conditions (Olson, 1991; Mokhtar, 2021).

However, changes in this elaborate structure can cause an impaired function. As the results of the present study suggest, even initial modifications such as the lifting of the simple squamous covering epithelium, usually associated with the formation of the subepithelial clefts, may be able to alter the countercurrent phenomenon. Due to a bigger distance between the lamellar axis and the exterior of the lamella, the oxygenated blood may find it harder to reach the destination point.

The countercurrent exchange represents a key adaptive mechanism that allows fish to extract oxygen efficiently even from waters with low oxygen concentrations. Because the direction of blood flow within the lamellae is opposite to that of water passing over the gill surface, a continuous diffusion gradient is maintained along the entire length of the exchange surface. This ensures that blood with the lowest oxygen concentration always encounters water with a higher oxygen concentration, maximizing diffusion potential (Genten *et al*., 2009). The efficiency of this mechanism critically depends on the structural and functional integrity of the lamellar epithelium and the associated vascular components (Mumford *et al*., 2007). Damage to these structures, even at the microscopic level, can have profound physiological consequences. Thickening of the epithelial layer, lamellar fusion, or disruption of pillar cell function increases diffusion distance and alters local hemodynamics, thereby compromising gas exchange and osmoregulatory balance (Mumford *et al*., 2007).

In normal conditions, oxygenated blood leaving the lamellae via efferent lamellar arteries is directed to the dorsal aorta for systemic distribution, while deoxygenated blood is continuously supplied from the ventral aorta through afferent branchial arteries. This circulation ensures that the gills serve as both a respiratory and a regulatory organ. However, because the epithelium is in direct contact with the external environment, it is also the first barrier exposed to potential aquatic contaminants such as heavy metals. The high permeability that benefits gas exchange also allows toxicants to penetrate the epithelial surface, initiating histopathological alterations that can impair countercurrent efficiency. Consequently, even subtle morphological changes such as epithelial hyperplasia, edema, or disruption of pillar cell organization can lead to significant reductions in oxygen uptake and ion exchange (Olson, 1991; Mumford *et al*., 2007; Yancheva *et al*., 2016). For the specimens analyzed in the present study, it is highly possible to have polluted water negatively affecting gill morphology. Moreover, the found vascular changes (such as the vasodilation of the lamellar axis, pillar cell structural changes and subsequently the presence of the aneurysms) were the most probable cause for an impaired countercurrent exchange. Similarly, vasodilation of the lamellar axis and disruption of pillar cell integrity have been linked to lamellar aneurysms in fish exposed to heavy metals such as cadmium (Thophon *et al*., 2003; Garcia-Santos *et al*., 2006). On the other hand, the presence of aneurysms can serve as a sensitive biomarker of acute copper exposure (Monteiro *et al*., 2005).

The earliest and most frequently observed lesion was interstitial or lamellar edema, accompanied by detachment of the lamellar epithelium. Such edema, repeatedly reported in fish exposed to heavy metals (Karlsson-Norggren *et al*., 1986; Hwang & Tsai, 1993; Sola *et al*., 1995; Cengiz & Ünlü, 2002; Pane *et al*., 2004; Cengiz, 2006; Nero *et al*., 2006; Velmurugan *et al*., 2007; Reid & McDonald, 1991), may represent an early response to increased vascular permeability and osmoregulatory stress. Lifting of the lamellar epithelium, often resulting from edema, may serve as a protective mechanism by increasing the diffusion distance that waterborne pollutants must cross to reach the bloodstream, thereby temporarily reducing metal uptake and acute toxicity (Mallatt, 1985; Thophon *et al*., 2003).

Another frequently observed alteration in our specimens was epithelial hyperplasia. This modification may be the result of a proliferative response, due to an adaptive mechanism. Such alterations were observed in various fish species exposed to trace metals (Arellano 1999; De Boeck *et al*., 2001; van Heerden *et al*., 2004; Figueiredo-Fernandes *et al*., 2007; Velmurugan *et al*., 2007; Mohamed, 2009; Georgieva *et al*., 2014). It is thought that hyperplasia and associated lamellar fusion effectively increase the water-blood barrier thickness and reduce xenobiotic absorption. However, these changes come at a physiological cost, as they diminish the respiratory surface area and impair both gas exchange and ion regulation (Yancheva *et al*., 2016).

Regarding the increased mucous secretion that was noticed in most of the analyzed samples, Georgieva *et al*. (2014), assumed that the increased cellularity leads to a thicker mucous layer that may act as an additional barrier against metal penetration (Georgieva *et al*., 2014).

CONCLUSIONS

Aquatic environment polluted with heavy metals may lead to lesions that compromise the pillar cell framework, disrupt blood flow through the lamellae, and severely impair the countercurrent exchange mechanism that underpins efficient oxygenation. The loss of lamellar structural integrity not only increases diffusion distances but also alters the precise alignment between water and blood flow, thereby reducing the gradient necessary for optimal gas exchange. In this context, the histopathological manifestations of metal toxicity, ranging from edema and hyperplasia to necrosis, represent both protective adaptations and detrimental outcomes that collectively undermine the physiological performance of the gills. Understanding these processes provides critical insight into how heavy metals disturb respiratory efficiency and countercurrent function, establishing gill histopathology as a powerful indicator of environmental contamination.

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MATEI-LATIU, M.C.; LATIU, C.; PAPUC, T.; MUNTEAN, G.C.; DAESCU, A.M.; RUS, V. & GAL, A.F. Cambios histopatológicos del sistema de contracorriente en branquias de peces constantemente expuestas en aguas contaminadas con metales pesados. *Int. J. Morphol., 44(3)*:1000-1007, 2026.

RESUMEN: Las branquias de los peces representan uno de los órganos diana más vulnerables a los contaminantes acuáticos. Numerosas investigaciones demuestran que la exposición crónica a metales pesados provoca lesiones estructurales y alteraciones en la regulación iónica. Sin embargo, hasta el momento, no está claro cómo estas alteraciones afectan el mecanismo de intercambio de contracorriente, esencial para la respiración de los peces. El presente estudio tuvo como objetivo describir exhaustivamente las respuestas histológicas y funcionales de las branquias a la exposición a metales pesados, explicando cómo estas modificaciones afectan el intercambio de contracorriente. Se recolectaron muestras pareadas de branquias de ejemplares adultos de *Squalius cephalus* (Linnaeus, 1758) procedentes de la cuenca del río Aries (área geográfica de Rosia Montana), un ecosistema acuático de alto riesgo donde la fauna está continuamente expuesta a concentraciones tóxicas de metales. Las muestras de branquias fijadas con formalina tamponada al 10 % se desmineralizaron, se procesaron con la técnica habitual de inclusión en parafina y se tiñeron con el método tricrómico de Goldner. Los resultados mostraron una gran cantidad de lesiones, que se clasificaron según su magnitud en cuatro clases diferentes: adaptativas, degenerativas, inflamatorias e irreversibles. Las tres primeras categorías estuvieron presentes en casi todos los individuos, mientras que la prevalencia de las lesiones irreversibles fue ligeramente menor. En estas condiciones, el mecanismo de intercambio contracorriente se ve afectado incluso por modificaciones iniciales como el levantamiento del epitelio de cobertura escamoso simple. Debido a una mayor distancia entre el eje lamelar y el exterior de la lamela, la sangre oxigenada puede tener mayor dificultad para llegar al punto de destino. En consecuencia, la pérdida de la integridad estructural lamelar reduce el gradiente necesario para un intercambio gaseoso óptimo, y tanto las adaptaciones protectoras como los efectos perjudiciales socavan colectivamente el mecanismo fisiológico de intercambio a contracorriente. Estos hallazgos demuestran que el daño histopatológico inducido por metales podría comprometer el intercambio gaseoso a contracorriente, proporcionando un marco de biomarcadores fiable para evaluar la toxicidad de metales pesados en el medio acuático.

PALABRAS CLAVE: Ecotoxicología acuática; Morfología branquial; Mecanismo fisiológico; Eficiencia respiratoria; Especies centinela.

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