Metformin-induced alterations in gills of the freshwater fish *Astyanax lacustris* (Lütken, 1875) detected by histological and scanning electron microscopy

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Abstract
The antidiabetic drug metformin is widely prescribed and found in different concentrations in the environment around the world, raising concern about potential impacts on aquatic life. Analyses of the effects of exposure of biological models to aquatic contaminants are important for assessing pollution effects on fish health. The gills of fishes represent primary targets of disturbance by pollutants, mainly because of the large surface of the respiratory epithelium and the high perfusion rate, which both help the entry of pollutants into this tissue. In this context, the aim of this work was to use gill histological analyses biomarkers to evaluate the toxicity of metformin on aquatic environmental systems, by means of chronic exposure for 90 days of *Astyanax lacustris* (lambari), an ecologically important neotropical species that can be used as an environmental bioindicator. Histopathological analyses were performed using Light and Scanning Electron Microscopy. The main changes were lamellar fusion, telangiectasia hyperplasia and disappearance of microridges. The morphological changes observed possibly interfere with the gill physiology, indicating an unfavorable situation to the presence of metformin in the water, pointing to a concern that metformin may pose a risk to *Astyanax lacustris* and likely to other fish species, compromising the dynamics of the aquatic ecosystem as a whole.
Graphical abstract

Keywords Antidiabetic · Histopathology · Ecotoxicological biomarker · Respiratory epithelium

Introduction

Aquatic ecosystems are the main recipients of contaminants (Abhilash 2012; Patel et al. 2019). Many drugs consist of organic compounds that are not easily biodegraded, and these compounds are unaltered by conventional treatment in Water Treatment Plants (WTP) or Sewerage systems (WTS) before being destined public drinking water supply or discarded into the environment, respectively (Santos et al. 2010; Stuart et al. 2012). Different therapeutic classes of drugs, including hypoglycemic agents, analgesics, antibiotics, hormones and antihypertensives, have been detected in waste and surface water at variable concentrations (Shao et al. 2009; Ibáñez et al. 2013; Oosterhuis et al. 2013; Santos et al. 2013; Bradley et al. 2016). However, only a small number of pharmaceutical products found in wastewater treatment plant effluents have been studied for environmental impacts (Heberer 2002; Fatta-Kassinos et al. 2011). Among them, the antidiabetic drug metformin, belonging to the biguanide class, is among the most abundant pharmaceuticals being introduced into the environment (MacLaren et al. 2018), at up to six tons per year from individual WWTPs in urban areas (Crago et al. 2016). The human body does not metabolize metformin and, therefore, approximately 90% of the therapeutic dosage is excreted in its original form (Dumitrescu et al. 2015).

The presence of metformin in the environment can be attributed to widespread medical prescription for the prevention of chronic diseases, including diabetes mellitus type II, treatment of polycystic ovary syndrome and some cancers (Sahra et al. 2010; Johnson 2014; Saraei et al. 2019). Due to its widespread use and the inefficient treatment of effluents, metformin has become an aquatic contaminant of emerging concern by the scientific community due to its potential environmental risk.

Ecological risk studies for environmental contaminants are often based on values derived from NOEC (no observable effect concentration). This dose descriptor corresponds to the highest concentration tested for which there is no statistically significant difference in effect when compared to the control group in the long-term ecotoxicity analysis (Caldwell et al. 2019). The NOEC value for metformin in chronic tests with zebrafish is 10,000 μg/L. (Jacob et al. 2018).

The influence of metformin on the aquatic environment has been demonstrated clearly in various studies, with adverse effects on different organisms, including bacteria, algae, cnidarians, crustaceans, fishes and amphibians.
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(Ambrosio-Albuquerque et al. 2021). In addition, chlorination of water creates potentially toxic metformin byproducts. These byproducts have distinct detrimental effects on aquatic microalgae (Yuanzhen et al. 2022).

A global-scale study of pharmaceutical pollution in 258 rivers along 104 countries, detected metformin at over 50% of all the sampling sites in all continents, except Antarctica. The highest mean metformin concentrations were observed in South America at 5.14 μg/L (Wilkinson et al. 2022). From our surveys, only one study reported the presence of metformin in Brazilian waters and it was present in all samples and at a highest concentration of 4.47 μg/L (Quadra et al. 2021). Even higher concentrations have been detected by other authors in different water systems. The detected concentrations vary from 39 μg/L to 56 μg/L in surface water in the Netherlands (Oosterhuis et al. 2013), 57 μg/L to 129 μg/L raw wastewater in Germany (Scheurer et al. 2009, 2012; Trautwein and Kümerer 2011) and 720 μg/L in hospital sewers in the USA (Oliveira et al. 2015). In Germany, metformin has also been detected in seawater and drinking water, which demonstrates the absence of efficient degradation processes, both in oceanic environments and in drinking water treatment plants, suggesting the high persistence and wide distribution potential of this drug (Trautwein et al. 2014).

Studies on the impact of metformin on fishes have shown its effects in different tissues like gills, gonads, etc., gonads etc. In *Pimephales promelas*, metformin caused the development of intersex gonads and induction of vitellogenin expression, among other genes involved in estrogen biosynthesis, indicating its potential as an endocrine disruptor (Niemuth and Klaper 2015; Crago et al. 2016). In the species *Betta splendens* (beta fish), metformin caused changes in aggressive behavior, with the potential to affect male reproductive fitness and possibly affect species survival (Maclaren et al. 2018). Meador et al. (2018), investigated the effects of emerging contaminants, including metformin, on two fish species (*Oncorhynchus tshawytscha* and *Leptocottus armatus*), and observed that metformin can inhibit animal growth and alter some metabolic pathways.

Previous research to determine dose descriptors in ecotoxicological studies has been limited to macroscopic analysis such as hatching, survival, growth rates and swimming behavior in model species such as zebrafish (Moermond and Smit 2016; Godoy et al. 2018), a species native to the Asian continent. However, extrapolation of pharmacological effects from ‘model’ species to other fishes should not always be adopted in risk assessment because of differences in species susceptibility to response to pharmaceuticals as observed among fathead minnow, medaka and zebrafish. Thus, the use of native species in experiments is important because they can present different responses, being more or less sensitive than the zebrafish (Brown et al. 2014).

Although ecological risk assessments for metformin have been rated as low (Blair et al. 2013; Huber et al. 2016) to moderate (Quadra et al. 2021), other authors have pointed out that metformin is an emerging critical contaminant that should be monitored in the environment (Chinnaiyan et al. 2018; Besse and Garric 2008). We also have to consider that it is difficult to determine a general risk acceptability criterion, as each aquatic ecosystem has its own specificities, for example, the mixture of pharmaceutical composition.

This study is an important component in the evaluation and understanding of the effects on non-target organisms of a contaminant of environmental significance. Our main aim was to check morphologic alterations in the gills of *Astyanax lacustris*, a neotropical freshwater fish with wide distribution in the Americas, and often used as a model for experimental toxicological study due to its small size, ease of collection, and maintenance. In addition to its ecological relevance, the species is produced in hatcheries on a commercial scale. This wide availability makes it a great option for use in laboratory tests, in addition to its known potential as a bioindicator to determine if there is a relationship between metformin exposure and biological responses that may demonstrate risk to population sustainability.

**Material and methods**

**Obtaining, maintaining and exposing fishes to metformin**

Juvenile specimens of *Astyanax lacustris* (20 to 30 days after hatching) were obtained commercially from a Piracema Fish Farm - Maringá (PR). Fishes were kept for 1 week of acclimation before the start of the experiment. The animals were maintained in well-aerated 100 L tanks containing dechlorinated water, at room temperature (±25 °C) with a natural photoperiod (12 h light/12 h dark-cycle corresponding to mean day length under summer conditions) and provisioned daily with commercial microfloculated feed (Alcon® - Camboriú (SC). The specimens were distributed randomly into five groups (n = 50 fishes per group), at a density of 1–2 g fish/litre. One group was kept under control conditions only in dechlorinated water and the other four groups were exposed to metformin (CAS No. 1115–70–4 Sigma Aldrich) at concentrations of 50 μg/L, 100 μg/L, 1000 μg/L and 10,000 μg/L for 90 days, constituting a chronic test. Concentrations of 50 μg/L and 100 μg/L were established based on concentrations found and described in the literature for surface water. The concentrations of 10,000 μg/L correspond to the NOEC. Metformin replacement was performed twice a week, with the tank water being completely changed (Sharma et al. 2010). Water temperature, oxygen content, conductivity, and pH were measured during the experiments and were kept within the comfort range for
these fishes, so that these parameters would not influence the results. All methods were performed in accordance with the relevant guidelines and regulations such as CONCEA—Conselho Nacional de Controle de Experimentação Animal (2017) and OECD (2009).

Ethical statements and method of euthanasia

All experimental procedures were approved and certified by the Ethics Committee on the Use of Animals (CEUA—State University of Maringá, Paraná, Brazil) under decision number 6409140218. Fishes were humanely euthanized by administration of an overdose of anesthetic (clove oil/eugenol) dissolved in water (Fernandes et al. 2017).

Scanning electron microscopy

For analysis of gills under scanning electron microscopy, three individuals were randomly selected at each concentration of metformin. The gills were dissected and fixed in Bouin (7.5% picric acid, 2.5% formaldehyde, 0.5% acetic acid) for 24 h at room temperature. They were serially dehydrated, with alcohol (7.5%, 15%, 30%, 50%, 70%, 90%, and 100%), critically dried in a Leica CPD030 drier and covered with gold using a Shimadzu IC-50 metalizer. Analyses were performed using a scanning microscope Quanta 250-Fei at the Microscopy Center of the Research Support Centers Complex (COMCAP) at the State University of Maringá, Paraná, Brazil (Gigliolli et al. 2015). The height of the sample is 10 mm from the detector, accelerating voltage 15.00 KV and the spot was 3.0. The microscope interface program was an xT Microscope. The operator was blinded to treatment and the resultant images were randomized to avoid observer bias in the final evaluation. Qualitative analyses of the gill alterations were carried out. For the capture of microridge images, the primary lamellae were chosen in order to avoid divergence in the location of the tissue where the capture occurred.

Light microscopy

For light microscopy, five individuals were randomly selected at each concentration of metformin. The gills were dissected and fixed in Bouin’s solution for 24 h. Samples were dehydrated in a series of increasing concentrations of alcohol (70%, 80%, 90%, and 100%), cleared in xylene, embedded in histological paraffin, and cut into 6μm thick sections with a Leica RM 2250 microtome. The sections were stained with hematoxylin and eosin (HE) (Junqueira and Junqueira 1983). Analyses were performed on an Olympus CX31RBSFA photographic microscope according to the semiquantitative method proposed by Schwaiger et al. (1997). The magnification was 100 x/ 1,30 oil, type of immersion medium was microscopy immersion oil (Merck São Paulo—SP). The software was Axio Vision Rel. 4.6, with camera AxioCam MRv.

Quantitative analysis of branchial alterations

For morphological analysis and gill alterations, 30 random fields per animal were evaluated under a light microscope at a total magnification of ×40 (Olympus CX31RBSFA) according to the semiquantitative method proposed by Schwaiger et al. (1997). An increasing scale of a Histological Alteration Index (HAI) was used that quantified the degree of severity of the injuries as described by Mallatt (1985); Level 0 = no histological change; Level 2 = moderate and specific changes; Level 3 = severe and extensive alterations. The data obtained were analyzed for normality using the Kolmogorov-Smirnov test. Subsequently, they were subjected to analysis of variance one-way (ANOVA) followed by the post-test of Tukey. The significance level adopted was 5% and the results were expressed as mean ± standard error. The software package used was graphpad prism version 8.

Results

Scanning electron microscopy

In the control group, the gills had primary and secondary lamellae devoid of alterations (Fig. 1a). The epithelial surface of microridges is composed of polygonal cells (paved cells), with well-defined contours, similar to fingerprints, which characterizes the normal appearance of these folds (Fig. 1b). In the group exposed to 50 µg/L of metformin, hypertrophy of the primary lamellae was observed (Fig. 1c), and the microridges were visible but presented a differentiated shape (Fig. 1d). After exposure to 100 µg/L, hypertrophy was observed in the primary and secondary lamellae (Fig. 1e, g), as well as a decrease in microridges (Fig. 1f). At a concentration of 1000 µg/L, hypertrophy of the primary and secondary lamellae was also observed (Fig. 1g) and the microridges showed severe deformity (Fig. 1h). At the concentration of 10,000 µg/L, it was possible to observe greater hypertrophy of the primary and secondary lamina in relation to the control and the other treatments (Fig. 2a–f), total secondary lamellar fusion (Fig. 2b–d), primary lamellar fusion (Fig. 2e), and total loss of microridges (Fig. 2g, h).

Histological analysis

Histological analysis revealed changes such as telangiectasia (Fig. 3B, H), bleeding (Fig. 3B, C), primary lamellar
fusion (Fig. 3D), vacuolization (Fig. 3E), hyperplasia (Fig. 3F) and secondary lamellar fusion (Fig. 3G).

Analyzing the averages of the Mean Values of Changes (M.V.C.) it was possible to observe a dose-dependence relationship for hyperplasia (Fig. 4A) and lamellar fusion (Fig. 4B) alterations, while for telangiectasia this correlation was not observed (Fig. 4C).

When compared statistically, all concentrations tested showed a significant effect in relation to the control ($p > 0.05$). Changes such as bleeding, edema and vacuolation were only observed in the gills exposed to a concentration of 10,000 μg/L; therefore, they were only analyzed qualitatively.

**Discussion**

Anthropogenic contamination of aquatic ecosystems by a class of emerging pollutants, the pharmaceutical residues of widespread use, has been a subject of increasing concern in the international scientific community. Results of the present study make it evident that even reported environmental
concentrations of the drug metformin lead to cellular reactions in the gills of *A. lacustris*, which should be considered an indicator of their damage to fishes. Some structure remodeling in gills may represent adaptive strategies by which the fishes maintain physiological functions (Tkatcheva et al. 2004; Richards 2011), but the histopathological lesions, as described in the present study, show that fishes were harmed by the exposure to metformin.

The respiratory system is the principal interface between a fish and its aquatic environment. Thus, this organ is highly sensitive to chemicals in water and is considered the primary target organ for contaminants (Camargo and Martinez 2007). We observed several types of gill damage by metformin in our analyses, many of which are similar to that documented in fishes experimentally exposed to other contaminants including pharmaceuticals, heavy metals and pesticides (Thophon et al. 2003; Hassaninezhad et al. 2014; Rodrigues et al. 2017, Zafra-Lemos et al. 2021).

Various environmental pollutants are known to affect gill morphology, including alterations in the microridges, “fingerprint” patterned structures commonly found on the branchial surface of the fishes (Karlsson 1983; Eiras-Stofella

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**Fig. 2** Scanning electron microscopy of *Astyanax lacustris* gills exposed to the highest concentration of metformin. a–h PLH primary lamella hypertrophy, SLH secondary lamella hypertrophy, SLF secondary lamella fusion, PLF primary lamella fusion, MD microridges. Bars: a, b, d, e = 50 µm; c = 25 µm; f = 15 µm; h = 5 µm

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et al. 2001), including from the genus *Astyanax* (Lopes et al. 2017). An F-actin-based cytoskeleton is the underlying core structural component of those structures. The widespread distribution of microridges among various species and tissues indicates that they have important and common roles in healthy organisms (Eiras-Stofella et al. 2001; Depasquale
The function of these structures is not known, although several roles have been proposed, such as providing structural support to stabilize the enveloping mucus layer of epithelia and mechanical strength against abrasion (Sperry and Wassersug 1976; Wilson and Laurent 2002; Ba-Omar et al. 2014). They may even serve as a means of increasing the surface area of cells for absorption purposes (Lam et al. 2015) or possibly act as a stable reserve of pre-polymerized F-actin (Sharma et al. 2005).

The action of metformin on the cytoskeleton may be related to the fact that this drug at the cellular level is known to cause inhibition of Complex I of the mitochondrial respiratory chain, which results in a decline in ATP production (Faure et al. 2018). ATP decline may affect dissociations between cytoskeletal proteins, which could explain the decrease and loss of microridges as observed in our analyses (Gov and Safran 2004).

Other substances have also been reported to affect microridges. In *Danio rerio* exposed to non-ionized ammonia, this “fingerprint” patterned structure was lost. (Al-Zaidan et al. 2013). A similar effect was seen in fishes of the different Aphanius species exposed to 3 µg/l of deltamethrin (Al-Ghanbousi et al. 2012), *Channa punctata* exposed to trace metals (Pandey et al. 2008) and *Oreochromis* sp. exposed to lead ions (Aldoghachi et al. 2015).

In addition to changes in microridges, fusion and lamellar hypertrophy were the most common changes in metformin exposed *A. lacustris*. According to Skidmore and Tovell (1972), the increased adhesion between epithelial cells and the pillar cell support system, associated with the integrity of the secondary lamella structure, can lead to lamellar fusion, which could influence the gas exchange process in the gills, resulting in insufficient gas diffusion between the lamellae. On the other hand, it can also occur because of lamellar hyperplasia, due to the union of lamellar capillaries within a mass of hyperplastic epithelium (Rajbanshi and Gupta 1988) as a result of the adaptive biological responses to low-quality water and in an attempt to maintain their physiological functions (Laurent and Perry 1991; Pereira et al. 2013).

For Reis et al. (2009), the lamellar fusion observed in the gills of *Oreochromis niloticus* may have resulted from compensatory defense mechanisms, but which may compromise the branchial function, depending on the severity of the process. Takashima and Hibiya (1995), attributed the occurrence of mucous cell hyperplasia as a consequence of defense mechanisms that may compromise or due to an increase in cell and tissue functions caused by physiological changes (Takashima and Hibiya 1995; Mohamad et al. 2021). Lamellar hyperplasia is a long-term response of Malpighian cells, derived from the primary lamellae that accumulate on the anterior edge of the secondary lamella, known as “lubrication”. Eventually, the entire interlamellar space can be filled with new cells, as result, the respiratory area is greatly reduced (Roberts 2012; Sales et al. 2017).

Changes in membrane permeability may result in increased cytoplasmic volume, which explains the lamellar hypertrophy observed in this study (Roberts 2012). Additionally, considering that the ion regulation process is correlated with specific neuroendocrine responses and with fishes reproduction (Dolomatov et al. 2012; Gabilondo et al. 2021), the question we raise is whether the observed histological changes can affect ionic homeostasis causing endocrine disruption? Recently, studies reporting the endocrine disrupting effect of pollutants in fishes have increased (Grieshaber et al. 2018; Barra et al. 2021; Blazer

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**Fig. 4** Graphs demonstrating the mean values of change (M.V.C.) of alterations in the gills of *Astyanax lacustris* gills in relation to metformin concentration. A hyperplasia, B lamellar fusion, C Telangiectasia, p < 0.05 when compared to the control group.
et al. 2021). The action of metformin in causing intersexuality in fishes had already been described previously (Niemuth and Klaper 2015) and, in our analyses, intersex animals were also observed (data not yet published).

Other disorders found in our analysis were alterations involving changes in the circulatory system among them telangiectasia, edema and bleeding. These changes are associated with variations in blood flow in the gills and commonly appear soon after exposure to a toxic agent (Barisić et al. 2015). In the present study, telangiectasia was observed at all drug concentrations, with the highest concentration coinciding with the highest degree of alteration. Intralamellar edema and bleeding were observed at the highest concentration of metformin (10,000 μg/L). Likewise, these changes were observed in Squalius varharensis coming from rivers near mines in Macedonia (Barisić et al. 2015), as well as in Acanthopagrus latus exposed to mercury chloride at different concentrations (Hassaninezhad et al. 2014).

Pathological changes, as we have seen in the present work, are common symptoms of toxic effects in fishes caused by a wide variety of aquatic pollutants. The mechanism of metformin causing the effects observed in the gills of fishes is not known and needs to be investigated, as the gills serve as a basis for studies of human kidney function (Evans et al. 2005; Hwang and Chou 2013; Ito et al. 2014). In fact, studies have shown that metformin can have an adverse effect on the physiology of this organ in people with impaired kidney function (Hsu et al. 2017).

Unfortunately, the morphological pathologies observed in the gills of A. lacustris do not provide information about the effect of metformin on the physiological changes that occur in the gills of fishes. However, from the results obtained, we can conclude that assessing the ecological risk based on NOEC utilizing model species can underestimate the risk level posed by these environmental contaminants.

Understanding the effects of metformin exposure on wildlife populations is the ultimate goal of risk assessment, but to date, most data have been obtained from endpoints and bioassays standard with model species, whose data can be not representative of other species. Evaluating the capacity of populations to persist in polluted environments requires knowledge of more endpoints in response to metformin, to understand the capability of populations to adapt to harmful effects.

Conclusion

This study showed that metformin, in concentrations similar to found in nature and defined as NOEC for fishes, is an active pharmaceutical ingredient that produce several morphological alterations in A. lacustris gills. Gill histopathology proved to be a morphological approach that provides a way to identify damage caused by metformin and can be used as a biomarker for analyses of ignored effects in macroscopic studies. Despite the usefulness of metformin in the treatment of diabetes, it has been proven that it causes deterioration of the functional units of the respiratory system, so the potential toxicological effects of this drug on fishes should be taken into consideration. Monitoring and other studies must be carefully considered, since it is clear that metformin is a very common water contaminant, but research studies have not yet provided a full view of its effects on gills and how this relates to broader complications, such as effects on population sustainability. Given the importance of the respiratory organ and the degree of injuries observed, even at environmentally relevant concentrations, metformin is a substance that gives rise to a concern that could potentially impact populations.

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Author contributions All authors discussed the results and contributed to the final manuscript. LAB-C, PAB and ALBP-C conceived of the presented idea. Material preparation, data collection and analysis were performed by PAB, LL, RFM, and BRP. The manuscript was written by PAB and AASG with support from LAB-C, ALBP-C, EVSLM and IFM-R. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval Ethical approval for this study was obtained from Ethics Committee on the Use of Animals (CEUA—State University of Maringá, Paraná, Brazil) under decision number 6409140218.

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