ACTA BIOLÓGICA COLOMBIANA
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Facultad de Ciencias
Departamento de Biología
Sede Bogotá

UNIVERSIDAD NACIONAL DE COLOMBIA

Research article

Histopathological alterations in the preoptic area of the fish *Piaractus orinoquensis* exposed to sublethal concentrations of glyphosate

Alteraciones histopatológicas en el área preóptica del pez *Piaractus orinoquensis* expuesto a concentraciones subletales de glifosato

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Received: 12th September 2024. **Revised**: 30th October 2024. **Accepted**: 01st April 2025
Associate Editor: Omar Hernando Ávila-Poveda

Citation/ citar este artículo como: Gómez-Ramírez, E., Riaño-Quintero, C. A. and Baldisserotto, B. (2025). Histopathological alterations in the preoptic area of the native fish *Piaractus orinoquensis* exposed to sublethal concentrations of a glyphosate-based herbicide. *Acta Biol. Colomb.*, *30*(2), 116-126. https://doi.org/10.15446/abc.v30n2.115683

ABSTRACT

Glyphosate (GP) is commonly used to control weeds in extensive and transgenic crops, which has generated environmental, ecological, and public health risks. It has been demonstrated that the presence of GP in aquatic ecosystems can affect species such as the Cachama blanca (*Piaractus orinoquensis*), a native species that has the highest consumption rates in Colombia. Therefore, this work evaluates the effect of a commercial formulation of glyphosate on the preoptic area of *P. orinoquensis*. Juveniles were exposed to sublethal concentrations (0, 1, 3, and 5 mg/L) of Roundup Activo® for 30 days. Seventy-two fish were sacrificed (per sampling), and their brains were extracted and processed for transmission electron microscopy (TEM) and high-resolution optical microscopy (HROM). Alterations were identified in the anterior zone of the preoptic area, characterized by increased mast cells, vacuolization, and lipid accumulation. A higher number of mast cells was observed in the anterior parvocellular nucleus at 1 mg/L, suggesting a first localized inflammatory response in that region of the brain. This indicates that immune processes in *P. orinoquensis* may be modulated by xenobiotic exposure, generating different responses. Ultrastructural analysis revealed partial mast cell degranulation at all GP concentrations. The increase of mast cells in certain neuronal nuclei could influence alterations in reproductive behavior, affect homeostasis, and, consequently, modify the dynamics and adaptability of *P. orinoquensis* in the natural environment.

Keywords: Brain, herbicide, inflammation, mastocyte, toxicology.

RESUMEN

El glifosato (GP) se utiliza ampliamente para el control de arvenses en cultivos extensivos y transgénicos lo que ha generado problemas medioambientales, ecológicos y de salud pública. Se ha demostrado que la presencia de glifosato en ecosistemas acuáticos puede afectar a especies como la Cachama blanca (*Piaractus orinoquensis*), una especie nativa que lidera los índices de consumo en Colombia. Por ello, este trabajo evalúa el efecto de una presentación comercial de glifosato sobre el área preóptica de *P. orinoquensis*. Se expusieron juveniles a concentraciones subletales (0, 1, 3 y 5 mg/L) de Roundup Activo® durante 30 días. Setenta y dos peces fueron sacrificados en cada tiempo de muestreo, y sus cerebros fueron extraídos y procesados para microscopía electrónica de transmisión (MET) y microscopía óptica de alta resolución (MOAR). Se identificaron alteraciones en la zona anterior del área preóptica, caracterizadas por un aumento de mastocitos, vacuolización y acumulación lipídica. Se observó un mayor número de mastocitos en el núcleo parvocelular anterior a 1 mg/L lo que sugiere una primera respuesta inflamatoria localizada en esa región del cerebro. Esto indica que los procesos inmunitarios en *P. orinoquensis* pueden ser modulados por la exposición al xenobiótico, generando diferentes respuestas. El análisis ultraestructural mostró una degranulación parcial de los mastocitos en todas las concentraciones de GP. El aumento de mastocitos en determinados núcleos neuronales podría influir en alteraciones del comportamiento reproductivo, afectar la homeostasis y, en consecuencia, modificar la dinámica y adaptabilidad de *P. orinoquensis* en el medio natural.

Palabras clave: Cerebro, herbicida, inflamación, mastocito, toxicología.

INTRODUCTION

Glyphosate is a broad-spectrum herbicide available in numerous commercial formulations, with Roundup Activo® (Bayer AG) being the most extensively used trademark in Colombia. This product serves multiple agricultural purposes, including weed control in both conventional and transgenic crops, grain desiccation, and the spraying of illicit crops (Valbuena *et al.*, 2021). The Colombian Orinoquia region, known for its high agricultural productivity, has experienced extensive and continuous fumigation with commercial glyphosate formulations, increasing the risk of contaminating natural water bodies and affecting aquatic organisms inhabiting these ecosystems (Merino *et al.*, 2013;). Furthermore, glyphosate-based herbicides (GBHs) have been reported to exhibit significantly higher toxicity than glyphosate itself, with toxic effects on aquatic organisms magnified by up to 100 times (Mesnage *et al.*, 2019). Similar findings have been documented in fish, where the increased toxicity is primarily attributed to the presence of surfactants, such as polyoxyethylene amine (POEA) (Eslava-Mocha *et al.*, 2019).

The National Environment Council (CONAMA) of Brazil establishes a limit of 65 μg/L of glyphosate (GP) for surface waters; the United States Environmental Protection Agency (US EPA) sets a limit of 700 μg/L of GP in drinking water and the European Commission (EC) stipulates a maximum allowable concentration of 0.1 μg/L for any pesticide in drinking water (Thomas *et al.*, 2019). However, numerous studies indicate that concentrations exceed the upper limits established for GP (Lopes *et al.*, 2022).

After the application of herbicides in agricultural processes, GP is absorbed by crops and simultaneously penetrates the soil, where it binds to various molecules and breaks down into AMPA through bacterial activity (Tresnakova *et al.*, 2021). Over time, when the soil reaches its saturation capacity due to the recurrent application of herbicides, GP may be transported to water bodies through leaching (Lupi *et al.*, 2019). This process can alter the physical and chemical properties of water, which is reflected in changes in the cellular and biochemical biology of aquatic communities, such as amphibians and fish (López-Flórez *et al.*, 2023), leading to significant changes in their tissues, physiology, and behavior (Tresnakova *et al.*, 2021).

Residual concentrations of AMPA in water bodies may persist for 45 to 60 days (Annett *et al.*, 2014), and are 3 to 6 times more toxic and persistent than GP, contaminating various aquatic organisms, primarily fish, through the absorption of the xenobiotic via gills and the food chain (Sun *et al.*, 2019).

Although the Environmental Protection Agency (EPA) and the European Food Safety Authority (EFSA) report glyphosate-based compounds as non-neurotoxic, several studies highlight a potential neurotoxic effect of GP in various animal species, causing central nervous system effects such as increased oxidative stress, neuroinflammation, glutamate excitotoxicity, changes in neurotransmitter profiles, and behavioral alterations (Faria *et al.*, 2021).

Fish have been identified as effective bioindicators for assessing risks associated with environmental pollution, facilitating the early detection of potential problems (Lopes *et al.*, 2022). In most species, the brain features structures such as the olfactory bulbs (OB), telencephalic hemispheres (TH), optic lobes (OL), inferior lobes (IL), cerebellum (CE), and medulla oblongata (MO) (Obando-Bulla *et al.*, 2013). In this context, the preoptic area extends from the TH to the rostral hypothalamus (RH) and plays essential roles in processing visual information from the retina, controlling ocular motor reflexes, regulating circadian rhythms, as well as modulating sexual behavior and aggression (O'Connell *et al.*, 2013). However, despite the significance of these processes in various species, there are few studies focused on the brain of the white cachama (*Piaractus orinoquensis*).

Most toxicological studies on this herbicide in fish have focused on acute exposures, evaluating high concentrations over short periods (Braz-Mota *et al.*, 2015). While these studies are important for determining lethal concentrations (LC50) and developing toxicological models, such concentrations are rarely found in aquatic environments (Riaño *et al.*, 2019). For example, the 96-hour LC50 of glyphosate in Roundup® for *P. orinoquensis* fry was determined to be 97.47 mg/L (Eslava *et al.*, 2007), whereas the highest concentration reported in surface and groundwater in southeastern Brazil was 5.8 mg/L (Lima *et al.*, 2023). Other studies on tropical fish, such as the Cardinal Tetra (*Paracheirodon axelrodi*), evaluated concentrations of 1.3 and 5 mg/L, showing increased presence of mast cells in blood vessels and possible necrosis and/or apoptosis (Riaño *et al.*, 2019); increased oxidative stress, reflected by elevated lipid peroxidation levels in the brain (Faria *et al.*, 2022); energy imbalance, stress responses, inhibition of acetylcholinesterase (AChE), and physiological and endocrine disorders (Akter *et al.*, 2020). Therefore, it is imperative to conduct studies evaluating the effects of sublethal concentrations of glyphosate over prolonged periods, as this active compound can persist in water for up to 91 days and subsequently transform into other metabolites through photodegradation (Grunewald *et al.*, 2001).

P. orinoquensis is an effective bioindicator of water quality in tropical rivers because it is a native fish that inhabits

Table 1. Water quality parameters for each treatment through the 30 days of exposure of *P. orinoquensis* to glyphosate (Roundup Activo®).

	Glyphosate (mg/L)			
Parameter	**0 (control)**	**1**	**3**	**5**
TAN (mg/L)	0.36 ± 0.18[a]	0.42 ± 0.13[a]	0.42 ± 0.14[a]	0.59 ± 0.19[a]
Nitrite (mg/L)	0.04 ± 0.02[a]	0.09 ± 0.04[a]	0.08 ± 0.03[a]	0.18 ± 0.11[a]
pH	6.73 ± 0.31[a]	6.64 ± 0.65[a]	6.99 ± 0.17[a]	7.07 ± 0.18[a]
Temperature (°C)	29.13 ± 0.57[a]	29.32 ± 0.68[a]	29.58 ± 0.74[a]	29.48 ± 0.70[a]
Dissolved oxygen (mg/L)	4.08 ± 0.42[a]	4.12 ± 0.32[a]	4.18 ± 0.44[a]	4.44 ± 0.38[a]
EC (μS/cm)	219.33 ± 36.54[a]	214.87 ± 42.76[a]	211.77 ± 41.86[a]	219.6 ± 48.21[a]

Different superscript letters in the rows indicate significant differences between treatments.

water bodies near areas sprayed with this herbicide (Gómez-Ramirez, 2013). Additionally, various aspects of this species' basic biology are known, and it holds significant economic importance, the second most commercially fish species in Colombia (MADR, 2018). Although toxicological research on this species concerning glyphosate-based herbicides has been conducted, the focus has predominantly been on histopathological changes in organs such as gills, liver, spleen, and kidneys, with minimal attention to the brain (Braz-Mota *et al.*, 2015). The brain, specifically the preoptic area (PA), is vital in fish for regulating reproduction and gonadal maturation, integrating visual information, and managing key circadian processes essential for population dynamics and species survival (Cerdá-Reverter and Canosa, 2009). Additionally, an increase in mast cells has been observed in the nervous system of neotropical fish *Paracheirodon axelrodi* exposed to sublethal concentrations of glyphosate in Roundup Activo®, indicating an inflammatory response to toxic agents such as glyphosate, surfactant, or their synergistic effect (Riaño *et al.*, 2019). Therefore, determining the histopathological alterations in the neuronal nuclei of the PA and quantifying the number of mast cells provides insight into the functions that may be affected in an organism by a toxic agent. This study aims to determine the histopathological and ultrastructural alterations in the PA of juvenile white cachama (*P. Orinoquensis*) exposed to sublethal concentrations of Roundup Activo®, and to quantify mast cells in PA neuronal nuclei.

MATERIAL AND METHODS

Maintenance of *P. orinoquensis*

Juveniles were obtained from specialized aquaculture companies. The fish were maintained in 20 L aquariums at a density of 10 fish per aquarium in semi-static systems with continuous aeration and without a biofilter; the photoperiod was 12 hours light and 12 hours dark. Physicochemical parameters, such as temperature, pH, electrical conductivity, and dissolved oxygen, were measured daily using a Hanna HI9829 multiparameter probe. Total ammoniacal nitrogen (TAN) and nitrite were measured with a Spectroquant® Multy photometer using high-sensitivity tests (Table 1). The fish were fed Tetracolor® (47.5 % crude protein), adjusted to 2 % of the total biomass, three times daily (8:00, 12:00, and 16:00 h) and water renewal of 50 % every 24h.

Experimental design

144 fish were exposed to different environmental concentrations of glyphosate (4X3): 0 (control), 1, 3, and 5 mg/L with three replicates for 30 days. For each experimental group, the volume of glyphosate (Roundup Activo®; RA; CAS: 1071-83-6) required to reach the final concentration was calculated. To calculate the volume of Roundup Activo®, the concentration of glyphosate in the form of potassium acid salt present in the commercial product (446 g/L) was considered.

At 0 and 30 days of exposure, 72 fish were sedated at each sampling time following as closely as possible the recommendations of the bioethical management of fish in research and biomedical research with animals (Underwood and Anthony, 2020) and in accordance with Colombian law (see, resolution number 8430 of 1993) (Ministerio de Salud, 1993). Therefore, fish were sedated with benzocaine (0.5 g/L), and after 10 min, spinal cord was sectioned as indicated by Gómez-Ramirez (2013). The PA was obtained by microdissection; three brains from each treatment were processed for high-resolution optical microscopy (HROM) and three others for transmission electron microscopy (TEM). No significant alterations were observed in the samples corresponding to time zero, so these data were not included in the analyses and results of this study. This is because the initial conditions did not show relevant variations that would justify their inclusion in the interpretation of the effects derived from glyphosate exposure, thus ensuring the validity and focus of the results obtained after the experimental exposure period.

Processing for HROM and TEM

The samples were fixed in 2.5 % glutaraldehyde for three days. Then, a wash with phosphate buffer (PB) was performed, and they were post-fixed in 2 % osmium tetroxide for 2 h. The samples were washed with PB and dehydrated in increasing concentrations of ethanol (50, 70, 90, and 100 %); each wash lasted 10 min. Infiltration was carried out starting with three 15-min washes in homogeneous mixtures of Poly/Bed 812® resin and acetone at increasing concentrations: 1:2, 1:1, and 2:1. Subsequently, imbibition in pure resin was carried out for 2 h, followed by inclusion and polymerization for 18 h in an incubator at 70 °C (Riaño *et al.*, 2019). For HROM, 1 µm thick sections were obtained with a Slee Cut 4060 rotary microtome and stained with toluidine blue (Gómez-Ramirez, 2013). Once the permanent mounts were obtained in HROM, the images were captured using an optical microscope (ZEISS) equipped with an Axiocam digital camera (ZEISS). The calibration of the photographs was carried out with the ImageJ Software (https://imagej.nih.gov/ij/download.html). For TEM, 0.1 µm thick sections were made with a Leica EM UC7 ultramicrotome. Once the sections were obtained, they were contrasted with lead citrate/uranyl acetate for 3 min each, then the sections were examined on a Jeol JEM 1400 Plus 120 kV transmission electron microscope. Photographs were acquired with a Gatan camera using Gatan Microscopy Suite software.

Statistical analysis

The mast cell count was performed by observing 180 slides per treatment; each slide had 8 slices of 1 µm thickness, for a total of approximately 480 µm observed for each brain. To avoid double-counting cells, fields were sampled at 8 µm intervals. The Shapiro-Wilk test was performed to evaluate the normality of the data, Levene's test to evaluate the homogeneity of variances, and the Durbin-Watson test to establish the independence of residuals. A completely randomized, fixed effects, balanced design was used. ANOVA technique was applied to compare the variances between treatments regarding the number of mast cells with a level of significance of 5% ($p < 0.05$ type I error) using the statistical software R Project (www.r-project.org). For the mast cell count, each neuronal nucleus was evaluated, considering the topological division of the PA (Obando *et al.*, 2013). Subsequently, the measurement was made in µm2 for each nucleus using the polygon selection tool of ImageJ version 1.53e. A relationship was found between the number of mast cells in each neuronal nucleus and nucleus area.

RESULTS

The exploration of the entire PA (approx. 2,500 µm) showed that the mast cells and histopathological alterations were concentrated only in the rostral area corresponding to the telencephalic hemispheres (approx. 1,200 µm). Therefore, only these brain areas were analyzed. The main histopathological finding in HROM was the presence of mast cells adjacent to both blood vessels and capillaries that supply the telencephalic parenchyma (Figs. 1A, 1B, and 1C). In the observation of the preoptic area (PA), mast cells were found only in the middle area of the telencephalic hemispheres, mainly in the neuronal nuclei present in the dorsal, middle, and dorsolateral areas located in the rostral area of the PA (Figs. 1A, 1B, and 1C). No alterations were found in the caudal areas of the PA close to the hypothalamic nuclei and optic tectum (Figs. 1D and 1E). Ultrastructural alterations were found in all treatments with Roundup Activo®. In fish exposed to 1 mg/L of glyphosate, mast cells with slight reticulation of granular content were observed, and significant differences were noted compared to the control group and other glyphosate treatment concentrations. In fish exposed to 3 and 5 mg/L, vacuolations, nuclear smearing, and lipid accumulation were also observed (Fig. 2).

No mast cells immersed in the parenchyma of the PA were found in control fish. However, fish exposed to 1 mg/L glyphosate showed a higher number of mast cells than those kept at 3 mg/L and 5 mg/L glyphosate. The nuclei with the highest number of mast cells were PPa and Dc in fish at 1 mg/L glyphosate. However, considering the area (μm^2) of the neuronal nucleus in relation to the number of mast cells (area/MC), PPa and Dl have the highest number of mast cells, followed by Dc. Similarly, the area-normalized mast cell count (area/MC) was higher in fish at 1 mg/L glyphosate. On the other hand, the Dm and ENv nuclei exhibited the lowest number of mast cells (Table 2).

DISCUSSION

The water quality parameters remained within the ranges that allow significant growth of *P. orinoquensis* in recirculation systems, according to Poleo *et al.* (2011). In the current study, dissolved oxygen levels and temperature were maintained at 4 mg/L and 28 °C on average, respectively, which indicates that these parameters were within the appropriate ranges to guarantee the maintenance of this species without observable physiological stress. The values of TAN, nitrite, electrical conductivity, and pH were within the water quality criteria for rearing fish in recirculating systems proposed by Colt (2006). Consequently, it can be assumed that the observed alterations were due to Roundup Activo®.

Several of the neuronal nuclei described in other teleosts, such as *Danio rerio*, *Paracheirodon axelrodi*, and *Carassius auratus*, were identified in the PA (Wullimann *et al.,* 1996; Obando *et al.,* 2013). The staining properties are important for the identification of neuronal nuclei and for determining possible cellular alterations (Obando *et al.,* 2013). In this aspect, the neurons of the PPa presented staining with greater intensity than in the Pm or the Dm; however, these differences in the staining of neurons within the PA were normal, and apparently, none of the morphological properties of the PA were affected by glyphosate exposure.

The increase in the number of mast cells in the telencephalic region of the PA of *P. orinoquensis* exposed to

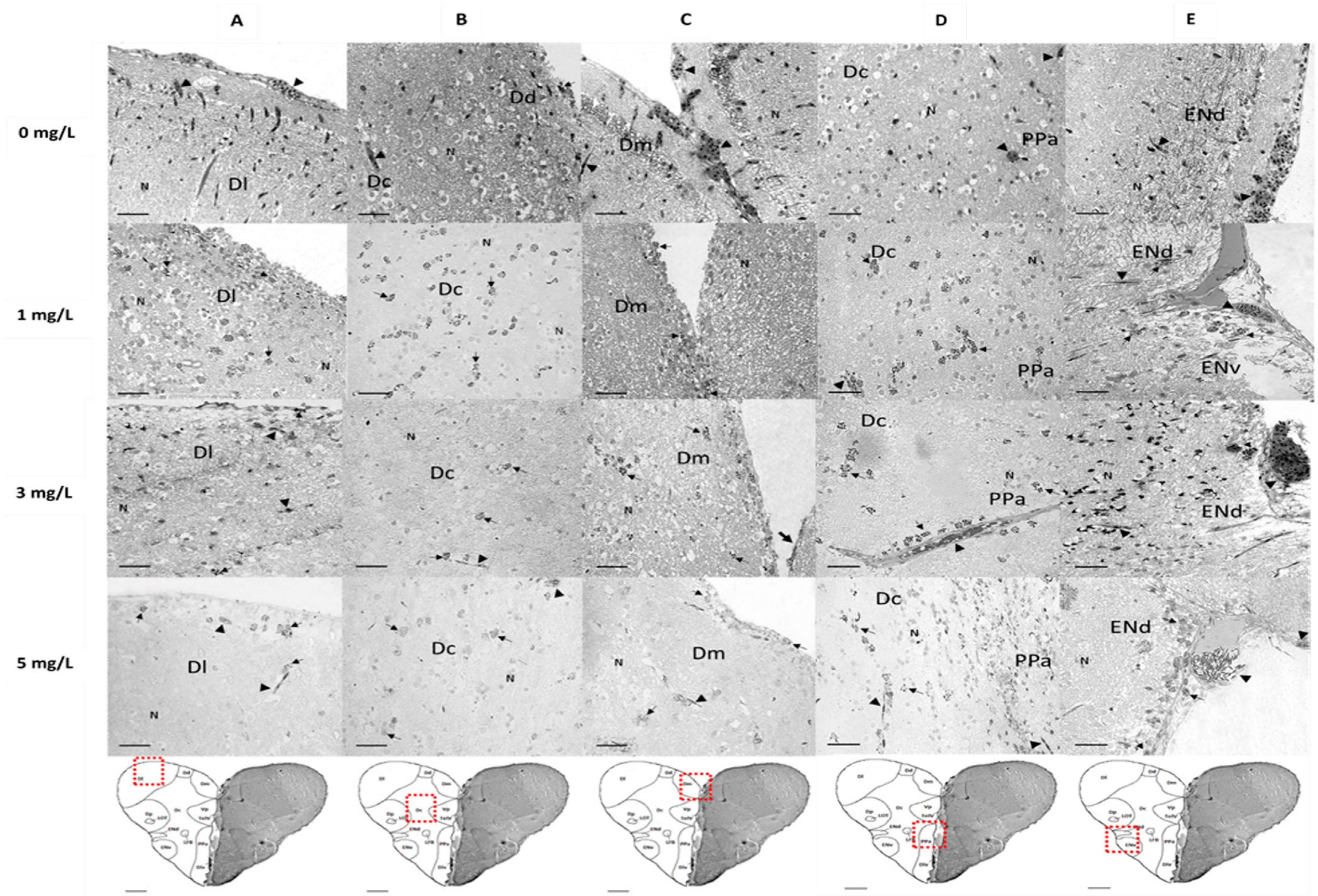

Figure 1. Mast cells found in different nuclei of the preoptic area (PA) in different zones of the telencephalic hemispheres (red boxes) of *P. orinoquensis* exposed to different glyphosate levels. A. Detail of the dorsal area of the dorsal telencephalon (Dl). B. Detail of the central dorsal telencephalic area (Dc). C. Detail of the middle zone of the dorsal telencephalon (Dm). D. Detail of the anterior parvocellular preoptic nucleus (PPa). E. Detail of the ventral entopeduncular nucleus (ENv) and dorsal entopeduncular nucleus (ENd). Adjacent blood vessels and capillaries correspond to arrowheads, mast cells correspond to black arrows, and N represents neurons. 25 μm bar for images of histology slides and 100 μm bar for the images of the topological division of AP. HROM, stained toluidine blue.

Roundup Activo® main histopathological finding with both histological techniques. Morphometry also supported this finding, with a higher number of mast cells in the PA. The mast cells have granules that contain a wide variety of contents, among which is heparin sulfate, which makes them visible using a metachromatic stain, such as toluidine blue, which reacts with the content of the granules forming a blue or dark purple color (Reite and Evensen, 2006). Their location is mainly perivascular, and they tend to proliferate when the organism is under chronic stress (Powell *et al.,* 1993), as is the case of *P. orinoquensis* exposed to this herbicide. In addition, the finding of mast cells in the rostral area of the PA, that is, in the telencephalic hemispheres and not in the caudal areas, may be related to one of the Roundup Activo® (or one of its compounds) entry routes that starts from the nostrils and extends through the olfactory rosette, olfactory bulbs, and telencephalon (Castañeda *et al.*, 2016). Probably, the exposure time allowed the diffusion of the xenobiotic only up to the telencephalic hemispheres following this entry route and not beyond it. It is probable that, at a longer exposure time, Roundup Activo® and its adjuvants could enter more caudal zones in the brain, taking advantage of the sessile arrangement of the olfactory bulbs and the telencephalic hemispheres; however, other studies would be needed to confirm this hypothesis.

Mast cells are part of the innate and adaptive immune system, and their main function is to promote the inflammatory process in the face of a stressful agent (Reite and Evensen, 2006). Although the content of the granules is diverse, in general, they possess vasodilator substances, such as histamine, serotonin, cytokines, and proteolytic enzymes (Theoharides and Cochran, 2004). Additionally, mast cells are involved in communication with other cells of the immune system, serving as mediators or indirect activators (Matsuyama and Iida, 1999). These cells also contain other substances, such as tumor necrosis factor (TNF), piscidins, alkaline and acid phosphatase, and nerve growth factor (NGF), among others (Theoharides and Cochran, 2004; Likewise, it has been reported that mast cells can secrete proinflammatory substances and signaling molecules without degranulation, indicating that only the

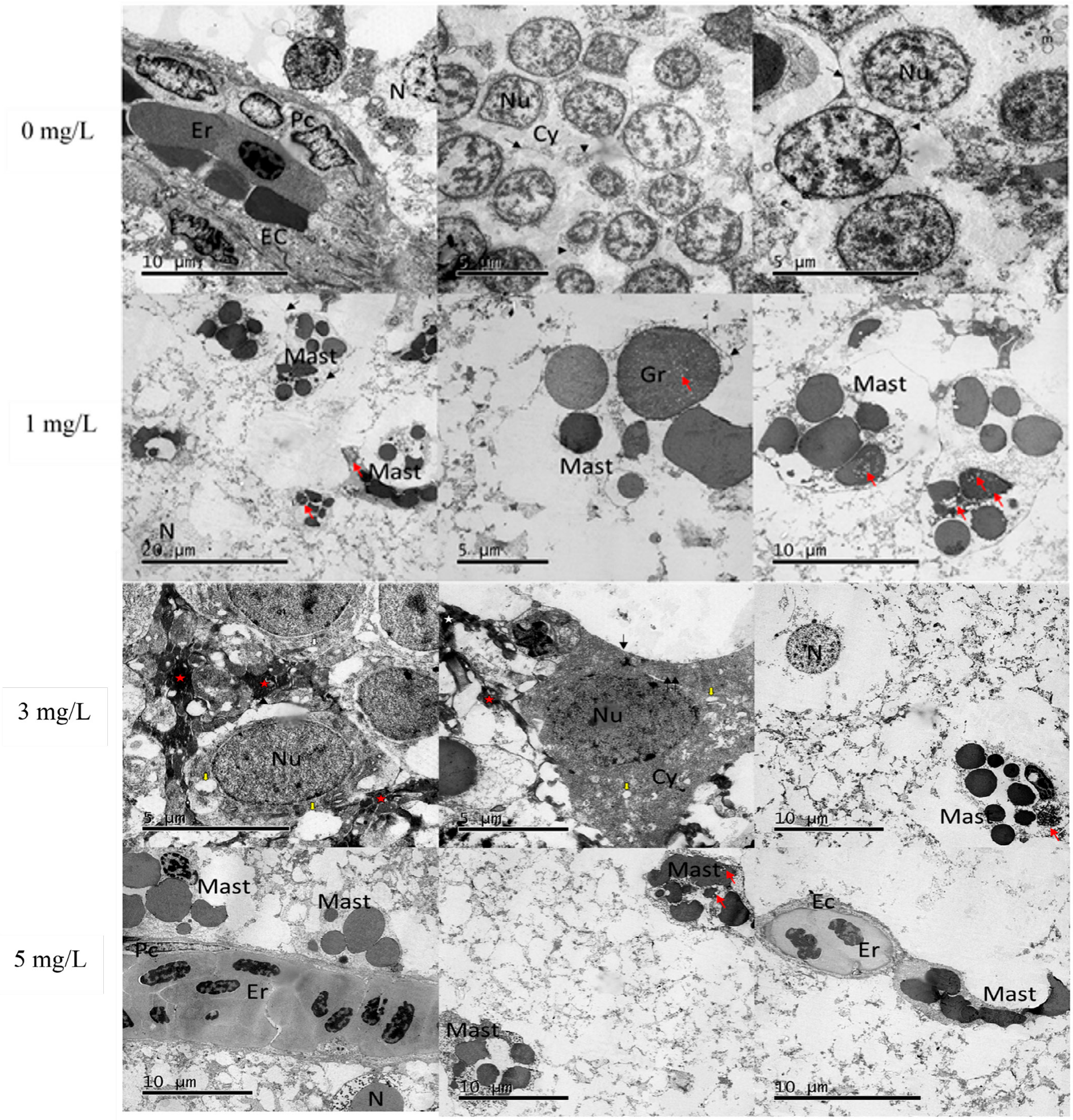

Figure 2. Ultrastructural alterations found in the preoptic area (PA) of *P. orinoquensis* exposed to different glyphosate levels using the transmission electron microscopy (TEM) technique. A capillary with erythrocytes (Er), pericyte (Pc), and an endothelial cell (EC) is observed in detail, as well as some neurons (N) with their cytoplasm (Cy), cell membrane (black arrows), and some ribosome pools (arrowhead) in the control fish (0 mg/L). Mast cells (Mast) with electron-dense granules (Gr) inside, neuron nuclei (Nu), vacuoles (yellow arrows), lipid accumulation (red stars), and details of some mitochondria (double arrow) and reticulation of the granular content (red arrows) are evident in fish exposed to all glyphosate concentrations.

presence of these cells can trigger an immune response in the presence of a stressful agent (Theoharides *et al.*, 2012).

Therefore, the increased proliferation of mast cells in the telencephalic area of the PA shows that the xenobiotic promotes the innate immune response, which is related to a defense reaction of the organism against a stressor agent, such as Roundup Activo®. The statistical analysis and the mast cell count for each treatment confirm this response and showed that, depending on the concentration of the xenobiotic, the number of mast cells changed. For example, the mast cell count was significantly higher in fish exposed to 1 mg/L glyphosate than in those from the 3 and 5 mg/L treatments. This suggests that the immune processes in *P. orinoquensis* can be modulated by exposure to the xenobiotic, generating different forms of responses depending, in this case, on its concentration.

This agrees with what was reported by Chen *et al.* (2009) and Sandig and Bulfone (2012), who found that the expression of genes encoding Toll-like receptors (TLR) and Nod-like receptors (NLR) was related to sublethal concentrations of Roundup GC®, showing higher expression at 0.5 mg/L than at higher concentrations. These receptors are found on various immune cells, but mainly on mast cells, and are important in the regulation of the innate immune response and inflammatory processes (Chen *et al.*, 2009; Sandig and Bulfone, 2012). These differences in the immune response are associated with compensatory cellular stress processes, in which low concentrations of the stressor agent allow the organism to activate survival mechanisms, but at higher concentrations, it triggers cell death processes (Uren and Santos, 2015). This is consistent with the finding of neurons in a possible necrotic process in fish exposed to 3 mg/L glyphosate, in which a significant decrease in mast cell proliferation was also seen.

The neuronal nuclei of the telencephalic zone of the PA have different functions in teleosts, so identifying alterations in a specific nucleus allows us to speculate about possible physiological effects in the species. For example, the Dl, Dm, and Dc telencephalon form a homologous neuronal complex of the pallial area in higher vertebrates. Dl presents abundant NMDA (N-methyl-D-aspartate receptor) synaptic receptors involved in learning processes, memory, and spatial representations of the environment (Gomez *et al.*, 2006) and receives visual information from a homologue of the pre-thalamic nucleus found in the ventrolateral diencephalon, forming an information transport neural network (Northcutt, 2008;).

Dc and Dm have a close integrative relationship with other neuronal nuclei involved in the reception of acoustic, olfactory, somatosensory, visual, gustatory, electro- and mechano-receptive, and lateral line information. They are also related to the stimulation of other areas of the PA, eliciting the release of sperm and mediating social, courtship, aggressive, and escape responses (Northcutt, 2008). In these nuclei, specifically in Dc, Dl, and Dm, an area/number of mast cells ratio of 0.16, 0.21, and 0.03, respectively, was found at the lowest glyphosate concentration (1 mg/L). The foregoing supports the idea that at the minimum concentration evaluated, it causes a strong activation of the immune system and does not lead to necrotic processes as was observed at concentrations of 3 and 5 mg/L of glyphosate.

The PPa in *P. orinoquensis* exposed to 1 mg/L glyphosate presented the highest value area/number of mast cells with respect to the other neuronal nuclei evaluated, reaching a maximum of 0.25. The PPa performs important

Table 2. Relationship (%) between the number of mast cells (MC) and the area of each neuronal nucleus of *P. orinoquensis* exposed to different glyphosate concentrations. Number of mast cells (MC) as mean ± standard deviation. Anterior parvocellular preoptic nucleus (PPa); dorsal telencephalic central zone (Dc); dorsal telencephalic dorsal zone (Dl); dorsal telencephalic midzone (Dm); ventral entopeduncular nucleus (ENv).

	Glyphosate (mg/L)					
	1 mg/L		3 mg/L		5 mg/L	
Neuronal nucleus and area	% Area/MC	Number MC	% Area/MC	Number MC	%Area/MC	Number MC
PPa (21,508 μm^2)	0.25	53.22 ± 17.05	0.15	31.57 ± 14.81	0.19	40.18 ± 11.83
Dc (38,873 μm^2)	0.16	79.83 ± 25.58	0.07	36.08 ± 16.92	0.12	60.27 ± 17.75
Dl (48,741 μm^2)	0.21	26.61 ± 8.52	0.10	13.53 ± 6.34	0.16	20.09 ± 5.91
Dm (12,957 μm^2)	0.03	10.64 ± 3.41	0.01	5.41 ± 2.53	0.02	8.03 ± 2.36
ENv (9,323 μm^2)	0.08	7.09 ± 2.27	0.04	3.06 ± 1.69	0.06	5.35 ± 1.57

Number of mast cells (MC) as mean ± standard deviation. Anterior parvocellular preoptic nucleus (PPa); dorsal telencephalic central zone (Dc); dorsal telencephalic dorsal zone (Dl); dorsal telencephalic midzone (Dm); ventral entopeduncular nucleus (ENv).

neuroendocrine functions, such as regulation of gonadal maturation, reproduction, and reproductive behavior (Bernier *et al.*, 2009), regulation of metabolism, visceral motility, release of gonadotropins, corticotropins and growth hormone, control of cardiovascular processes and blood pressure, release of gastric acids, modulating the activity of adenohypophyseal cells, and participation in the control of osmoregulation (Cerdá-Reverter and Canosa, 2009). This is an important finding, since alterations in this neuronal nucleus could trigger a systemic metabolic imbalance, mainly affecting reproduction, digestive processes, and osmoregulation. However, more studies are needed to determine if this species can reverse the harmful effects of Roundup Activo® and if it can mature and reproduce normally when reaching the minimum size of maturity. To enhance these studies, it would be beneficial to include behavioral assays, such as the Novel Tank test, in order to more accurately assess whether glyphosate impacts reproductive behavior, population dynamics, or survival ability. Additionally, immunohistochemical analysis of cortisol and caspase, which identify stress and necrosis processes, would contribute to a more detailed understanding of the physiological and molecular effects of glyphosate. Incorporating these methodologies would facilitate a more comprehensive evaluation of the underlying mechanisms of the alterations observed in the exposed organisms.

Mast cells with reticulated granular content were found in fish exposed to all concentrations of Roundup Activo®, showing a moderate degranulation process. This agrees with Schmale *et al.,* (2004), who induced degranulation in teleost mast cells by exposing them to stressful substances, such as capsaicin, compound 48/80, and substance P. This is important because in HROM it was not possible to observe this process and it was reported as absent; however, by means of TEM, the moderate degranulation of mast cells was evidenced as a possible process of immunological activation triggered by sublethal concentrations of Roundup Activo®.

Chronic exposure of different species of teleosts to some neurotoxins, organophosphate insecticides, and water contaminated with industrial waste provokes some ultrastructural changes in neurons that include vacuolization, edema, loss of membrane integrity, lipid accumulation, chromatin condensation, increased cytoplasmic transparency, and binucleate nuclei, among others (Lakshmaiah, 2017). This agrees with some of the findings observed in TEM for *P. orinoquensis* exposed to Roundup Activo®, especially in the treatment with 3 mg/L where vacuolization, nuclear smearing, and lipid accumulation were observed.

These reports indicate that the observed cellular alterations are typical of neuronal necrosis. This supports the possibility that the herbicide induces compensatory cellular stress, in which immune processes and cell proliferation are reduced and cell death pathways are promoted. This could have a negative impact on this species, because of the multiple functions of PA that could cause the partial loss of several key processes for the survival of this species. Among those that stand out are the loss of the integration of visual information, changes in the secretion of hormones, social behavior, and osmoregulatory imbalance, among others.

The use of glyphosate-based herbicides (GP) impacts water quality and non-target organisms. In fish, histopathological changes have been observed in the gills, such as proliferation of filamentous cells and cellular hyperplasia, as well as vacuolization in primary tissues such as the liver and brain, and nuclear pyknosis in the liver and kidneys. Additionally, alterations in enzymatic parameters, including acetylcholinesterase, butyrylcholinesterase, carboxylesterase, and glutathione S-transferase (GST), have been reported, along with changes in sexual activity and transaminases. Metabolic, hematological, and biochemical alterations in various organs have also been recorded, along with changes in tissue constituents, such as total lipids and glucose, among others (Lajmanovich *et al.*, 2011).

Cytoplasmic vacuolization results from an increase in intracellular water, mediated by the alteration of membrane function, and occurs when there is denaturation of the ATPases responsible for regulating cell volume or when the energy transfer processes required for cellular ionic regulation are disrupted (Abdelhalim and Jarrar, 2012).

Although sublethal concentrations were evaluated in this work, there are no established environmental reference values for Colombia. However, it is important to identify the effects that GBH have on native species, such as *P. orinoquensis*, since in a probable scenario of water source contamination, negative effects can be projected on natural and captive populations, which maintain farming systems that represent an important sector in the aquaculture market in this region of the country.

The main histopathological alteration found by HROM in the PA was the presence of mast cells. Mast cells were mainly concentrated in the dorsal telencephalic region of the PA, distributed in neuronal nuclei of the Dl, Dm, Dc, PPa, ENv, and ENd. The area that presented the highest number of mast cells was the PPa in fish from the 1 mg/L treatment. The ultrastructure findings showed that the mast cells partially degranulated in fish exposed to all Roundup Active® treatments; likewise, signs of possible necrosis were observed in some neurons.

CONCLUSIONS

This study demonstrates the importance of studying the effect of sublethal concentrations of glyphosate-based herbicides on native fish species. Although 100 % survival was obtained, damage was observed in brain regions that coordinate reproduction, migration and predator avoidance. Therefore, fish exposed to these concentrations in the natural environment could be more susceptible and

and could experience reduced fitness. In addition, this work provides relevant information for environmental authorities to regulate the use of these herbicides.

AUTHOR'S PARTICIPATION

E.G.R.: experimental design, data analysis, manuscript review, resource acquisition, project administration.

C.R.Q.: assembly of experimental units, maintenance, and fish intoxication, histological processing, image digitization, drafting of the initial manuscript.

B.B.: experimental design, data analysis, manuscript review

ACKNOWLEDGEMENTS

This study was supported by the vice-president for research of the Universidad Militar Nueva Granada in the project INV-CIAS 3146 and the Vice-Rectory of research of the Universidad Militar Nueva Granada - Validity 2021. Acknowledgments to Catalina López Flórez for proofreading the manuscript.

ETHICS STATEMENTS

This study was approved by the ethics committee of the Vice-Rectory of Research of the Universidad Militar Nueva Granada under INC-CIAS code 3146 and each fish was sedated before killing (Underwood and Anthony, 2020) following the bioethics recommendations for the humanitarian killing of animals established under the Colombian law (Resolution 8430 of 1993). The procedures for fish size selection, collection, relaxation, and sedation are consistent with the ethical considerations for field research on fishes and guidelines for the use of fishes in research (Jenkins *et al.*, 2014; Bennett *et al.*, 2016).

CONFLICT OF INTEREST

The authors declare that they have not conflict of interest.

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