

## Article

# Effect of Inorganic Mercury on Semen Quality, Embryo and Larval Development of Bocachico *Prochilodus magdalenae*

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**Abstract:** The study aimed to evaluate the effect of inorganic mercury (iHg) on the sperm quality, embryos, and larvae of *Prochilodus magdalenae*. Two experiments were carried out. In experiment 1, semen was activated with contaminated water at different iHg concentrations (0.0, 25, 50, 100 µg/L) and sperm kinetics were analyzed with a computer-assisted semen analysis (CASA) system. In the second trial, 2 g of oocytes were fertilized with 50 µL of milt, activated with contaminated water at different iHg concentrations (0.0, 25, 50, 100 µg/L), and maintained that way during the first hour of incubation. Samples were taken every 3–4 h until hatching to identify embryo deformations. Fertilization and hatching rates were estimated. The survival of newly hatched larvae (1 day) and larvae eight days post-hatching (dph) was also assessed. The results show that the quality parameters of semen treated with contaminated water at different iHg concentrations (25, 50, 100 µg/L) were altered and showed significant differences ( $p < 0.05$ ) from the control group (0 µg/L). Total motility decreased (9.8–13.2%) and the amount of immotile sperm increased (3-fold) relative to values in the control group. A significant increase in oocyte diameter and a decrease in fertilization and hatching rates were observed with the higher iHg concentrations (50–100 µg/L). Embryo deformations (>40%) such as scoliosis, lordosis, and microcephaly were observed, as well as premature hatchings. The larval survival at 1 dph was reduced between 25% (25 µg/L) and 97.2% (100 µg/L) relative to that in the control group. The results suggest that bocachico semen, embryos, and larvae are sensitive to contamination with iHg from 25 µg/L.

**Keywords:** heavy metals; migratory fish; pollution; reproduction; spermatozoa

**Key Contribution:** The study demonstrated that for externally fertilizing fish, such as the bocachico *Prochilodus magdalenae*, when gametes are released in waters polluted with inorganic mercury (25–100 µg/L), the seminal quality is altered and the fertilizing capacity is decreased. In embryonic development, deformations such as scoliosis, lordosis and microcephaly are registered, and the survival of newly hatched larvae is reduced between 25% (25 µg/L) and 97% (100 µg/L). Bocachico is highly sensitive to the toxic effects of inorganic mercury in the early stages of its life cycle.



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## 1. Introduction

The main waste generated by gold mining is mercury (Hg). This heavy metal is considered highly toxic and ubiquitous, with diverse natural and anthropogenic sources [1]. Once discharged, mercury is dispersed through the air and stored in water, soil, and the atmosphere, redistributing among these environments depending on environmental parameters, emission source, and dumping site. In the soil, mercury has a long retention time; once accumulated, it continues to be released into surface waters and other environments for prolonged periods [2]. The contamination of the aquatic environment with heavy

metals threatens the recruitment and survival of aquatic fauna due to detrimental effects on metabolism, growth, and reproduction [3–7].

Colombia is one of the countries that pollutes the most with mercury, and in 2021 was considered the fourth largest gold producer in Latin America, with a production of 76.8 tons, representing an increase of 17% compared to production in 2020 [8]; it was also considered to hold the first place in per capita pollution in the world [9]. Concern about inappropriate practices and excessive use of mercury in informal and illegal mining and their threat to Colombian fish biodiversity is growing daily [10–13].

In Colombia, more than 100 tons of inorganic mercury is discharged into the Magdalena River annually [14], and the contamination by inorganic mercury in the waters of rivers such as the Magdalena (0.06–0.12 µg/L), Cauca (0.004–0.240 µg/L), and Nechí (0.0015–2.580 µg/L) [14,15] reaches concentrations that affect aquatic life. It is important to note that in the Cauca River, where gold mining occurs, direct discharge of mercury can reach very high concentrations that affect fish survival, mainly when it occurs in spawning and early development areas of the fish [15,16]. Likewise, there are records of mercury occurring in tissues of fish species from this basin such as *Hoplias malabaricus*, *Ageneiosus pardalis*, *Prochilodus magdalenae*, *Triporthus magdalenae*, and *Megaleporinus muyscorum* [17,18].

According to the USEPA [19], for aquatic life, Hg levels of 1.4 µg/L imply acute toxicity and 0.8 µg/L is considered chronic toxicity [20]; however, Boyd [21] points out that concentrations >0.1 µg/L in natural waters affect aquatic life.

Fish absorb heavy metals mainly through the gills, digestive tract, and, to a lesser extent, the skin. Toxicity depends on metallic form, speciation, bioavailability, toxicokinetics (absorption, distribution, biotransformation, and excretion), and toxicodynamics (interactions with ligands) [22]. Most of the Hg found in water, soil, sediments, and biota is in the form of inorganic Hg ( $\text{Hg}^{+2}$  or  $\text{Hg}_2^{2+}$ ) and organic Hg, particularly monomethylmercury (MeHg) and dimethylmercury (DMHg) [23–25]. The methylation of mercury is a key step in the entrance of mercury into food chains. The biotransformation of inorganic mercury species to methylated organic species in water bodies can occur in the sediment and in the water column [25]. Factors such as concentration, pH, temperature, presence of organic matter, microbial activity, dissolved oxygen, and type of sediments can affect the toxicity and speciation of mercury in the aquatic environment [13,24]. The intake of heavy metals by fish and accumulation in the organism result in alterations in the structure and function of different tissues and vital organs [26].

Vital fish organs like the gills, liver, and kidney are susceptible to heavy metal toxicants; these organs respond differently to various heavy metals and are considered important bio-monitoring tools in evaluating the toxic effects of mercury on different fish species [27–29]. Prolonged exposure to heavy metals induces cytotoxicity and causes degenerative changes in the vital organs of the fish [30]. Also, the exposure of fish to heavy metals is manifested by multiple changes in hematological (red blood cells, hemoglobin, leucocytes, and lymphocytes) and biochemical parameters (glucose, glycogen, cholesterol, albumin, transferrin, ceruloplasmin) [29–31].

In reproduction, the quality of the gametes plays a key role in fertilization and embryonic development [5]. Parameters such as total motility, sperm velocity, and the duration of motility are considered critical to the fertilizing capacity of semen [23,24]. So, exposure of sperm to toxic substances could affect its quality and fertilization capacity [32,33].

The fertilization capacity of fish that freely release their gametes into the environment (external fertilization), such as the bocachico *Prochilodus magdalenae*, can be affected by contaminants such as heavy metals [32]. Mercury affects the quality of spermatozoa and therefore impedes fertilization and embryo development, either by accumulation in the gonads or by exposure in the aquatic environment during external fertilization [33].

Externally fertilized fish, such as the bocachico, simultaneously release their gametes into the riverbed during spawning, and this can occur in mining areas with high discharges of inorganic mercury. The chorion does not fully protect the embryo against the penetration

of metals, particularly during the egg's hydration (swelling) phase; therefore, metals can accumulate in the egg [26,34,35]. During embryonic development, a strong influence of metals has been reported immediately after fertilization and during the hydration phase of the egg. In *Cyprinus carpio*, it was reported that the chorion is permeable to metal ions such as cadmium and lead, which can change the structure and permeability of the chorion [26]. The permeability of the chorion decreases markedly after hydration and fertilization [36,37]. In neotropical migratory fish, the hydration phase of the eggs takes between one and two hours after fertilization [38]. Heavy metals can affect embryonic development processes, resulting in reduced quantity and quality of offspring [26].

Fish embryonic and larval deformations disturb population dynamics because they affect survival, growth, well-being, morphology, and ability to interact with habitats [39,40]. Among the damages reported in fish by heavy metals are deformations in the spine (lordosis, scoliosis, kyphosis), cephalic region, fins, and lateral line [40,41].

*Prochilodus magdalenae*, commonly known as bocachico, is the main species of Colombian freshwater fisheries, with great importance for the food security of the riverside county inhabitants of the Magdalena, Cauca, Atrato, and Sinú rivers [42]. Catches of this species have drastically decreased from 40,000 tons in 1975 to 4517 tons in 2020 [43,44]. Due to the drastic decrease in its catches, it has been categorized as vulnerable to extinction [45]. It is a potamodromous species whose population dynamics depend on annual migrations and the interactions between the rivers where they reproduce and the swamps where they feed and grow [46]. This species spawns in the main channels of the rivers, where its embryo development also takes place; then, the larvae enter the flood plains (swamps) to continue their development and growth [47,48].

Mercury in gold mining is a general problem that could affect the availability, distribution, genetics, and development of native fish species and, therefore, human populations consuming contaminated fish [13,24]. This study aimed to evaluate the effect of inorganic mercury on semen quality, embryos, and larval development of bocachico to provide basic information for managing native fish species contaminated by this xenobiotic.

## 2. Materials and Methods

### 2.1. Semen and Egg Collection

The gametes were obtained by induced reproduction of two-year-old bocachico broodstock ( $0.35 \pm 0.5$  kg) kept in captivity at the Institute of Fishculture Research of the University of Córdoba—CINPIC (Montería, Colombia). The females were injected with carp pituitary extract at a dose of 5 mg/kg of live weight in two doses (10% and 90% of the total dose in 12 h), and the males received a single dose of 4 mg/kg of live weight [49]. The gametes were collected six hours after the second injection of the females [49].

### 2.2. Experiment 1

In a Makler chamber (Sefi Medical, Haifa, Israel), 0.25  $\mu$ L of semen was activated with 75  $\mu$ L of water contaminated with different concentrations of inorganic mercury ( $\text{Hg}^{2+}$ , iHg): 0.0, 25, 50, and 100  $\mu$ g/L. Total motility, types of motility (rapid, medium, and slow), progressivity, curvilinear velocity (VCL), and straight linear velocity (VSL) were evaluated with a computer-assisted sperm analysis (CASA) system (Microptic, SCA-Vet, Barcelona, Spain) and a contrast microscope of phase (Nikon, Eclipse 50i, Tokyo, Japan). Rapid motility was considered the percentage of spermatozoa with velocities greater than 100  $\mu$ m/s, medium motility described spermatozoa with velocities less than 100  $\mu$ m/s but greater than 50  $\mu$ m/s, and slow motility described the percentage of spermatozoa with velocities less than 50  $\mu$ m/s [49]. The duration of motility was estimated as the time until around 90% of the spermatozoa stopped moving [42]. In addition, the straightness index (STR), linearity index (LIN), oscillation index (WOB), and parameters of angularity and oscillation of the spermatozoa heads, such as lateral width of the head (ALH) and beat cross frequency (BCF), were estimated.

### 2.3. Experiment 2

Fertilization was performed *in vitro* by mixing 2 g of oocytes with 50  $\mu\text{L}$  of semen, which was activated and maintained during the first hour of incubation with water contaminated with different concentrations of inorganic mercury ( $\text{Hg}^{2+}$ , iHg) in the form of  $\text{Hg}(\text{NO}_3)_2$ : 0.0 (control) 25, 50, and 100  $\mu\text{g}/\text{L}$ . For the preparation of the contaminated water, a standard solution of mercury  $\text{Hg}(\text{NO}_3)_2$  (1000 mg/L Hg) (Certipur<sup>®</sup>, USA) in  $\text{HNO}_3$  (2 mol/L) was used. Aliquots of 50, 100, and 200  $\mu\text{L}$  of this standard solution were taken to prepare 2 L of solutions with concentrations of 25, 50, and 100  $\mu\text{g}/\text{L}$ ; distilled water was used to complete this volume and maintain the pH of these solutions between 6.5 and 7.5. The gametes were kept in the contaminated water at different iHg concentrations for one hour during the fertilized egg hydration period [43]. Then, the embryos were transferred to experimental incubators (2.5 L of volume) with an upflow of uncontaminated water until hatching, approximately 14 h post-fertilization (hpf).

In the incubation and larviculture water, iHg concentration was estimated, and iHg concentration in the oocytes after exposure to the different concentrations was evaluated. The iHg was estimated as total mercury using a direct mercury analyzer (Milestone, DMA-80, Sorisole, Italy) with the following sequence: thermal decomposition, catalytic conversion, amalgamation, and atomic absorption spectrophotometry (EPA 7473). Each measurement was performed in triplicate for each experimental unit (incubator).

### 2.4. Embryonic Deformations

Embryo samples ( $n = 50$ ) were taken in triplicate at 1 (cleavage), 5 (gastrula), 8 (segmentation), and 11 h post-fertilization (hpf) (pharyngula) to identify embryonic deformations in each incubator. Bocachico eggs and larvae were photographed with a stereoscope (Zeiss, Stemi 508, Jena, Germany) with a built-in camera (Canon, G10, Japan) and then measured with image analysis software (Zeiss, Axiovision 4.8, Jena, Germany).

### 2.5. Fertilization and Hatching Rates

The fertilization rate was estimated at 5 hpf (gastrulation stage), and the hatching rate was measured at 10 hpf (pharyngulation stage). Viable embryos were considered translucent, and non-viable embryos were opaque, with detachment of the cellular material and without a chorion (premature hatching). Fertilization and hatching rates were estimated by dividing the number of viable embryos by the total number of embryos analyzed [49].

### 2.6. Larval Survival

The newly hatched larvae from each experimental incubator were transferred to 5 L tanks with water exchange and permanent aeration. Larval survival was estimated on the first day post-hatching (1 dph) and at 8 dph based on the number of larvae obtained at 1 dph.

### 2.7. Water Quality

Twice during incubation (~14 h) and daily during larviculture (8 days), water quality parameters such as temperature, dissolved oxygen (YSI, 550A, Yellow Springs, OH, USA), and pH (YSI, 100, Yellow Springs, OH, USA) were measured. Likewise, hardness, total alkalinity, non-ionized ammonia, and nitrites were measured with a photometer (YSI, 9500, Yellow Springs, OH, USA). The temperature (26.0–27.6  $^{\circ}\text{C}$ ), dissolved oxygen (4.2–7.5 mg/L), pH (7.2–8.4), total alkalinity (40.0–70.0 mg  $\text{CaCO}_3/\text{L}$ ), total hardness (50.0–60.0 mg  $\text{CaCO}_3/\text{L}$ ), non-ionized ammonia (0.08–0.11 mg/L), and nitrite (0.02–0.04 mg/L) levels ranged within normal values for the embryonic and larval development of neotropical fish species [50].

### 2.8. Statistical Analysis

A completely randomized design was used, with four treatments evaluated in triplicate. All variables were submitted to normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene test) tests. The percentage data were normalized using the arcsine

square root transformation and then were analyzed using ANOVA followed by Tukey's multiple range test. In all cases,  $p < 0.05$  was considered statistically significant. The analysis was performed with the Statgraphics Centurion XVI program. All results were expressed as mean  $\pm$  standard deviation.

### 3. Results

#### 3.1. Semen Quality

Table 1 shows the semen quality of bocachico activated with water contaminated at different concentrations of iHg (0, 25, 50, and 100  $\mu\text{g/L}$ ). The results show that the quality parameters of semen treated with contaminated water at different concentrations of iHg (25, 50, and 100  $\mu\text{g/L}$ ) were altered and showed significant differences ( $p < 0.05$ ) from those of the control group (0  $\mu\text{g/L}$ ), except VSL, STR, and LIN. In addition, the values of the different parameters analyzed from semen activated with contaminated water at iHg concentrations from 25 to 100  $\mu\text{g/L}$  did not show statistical differences among these treatments ( $p > 0.05$ ).

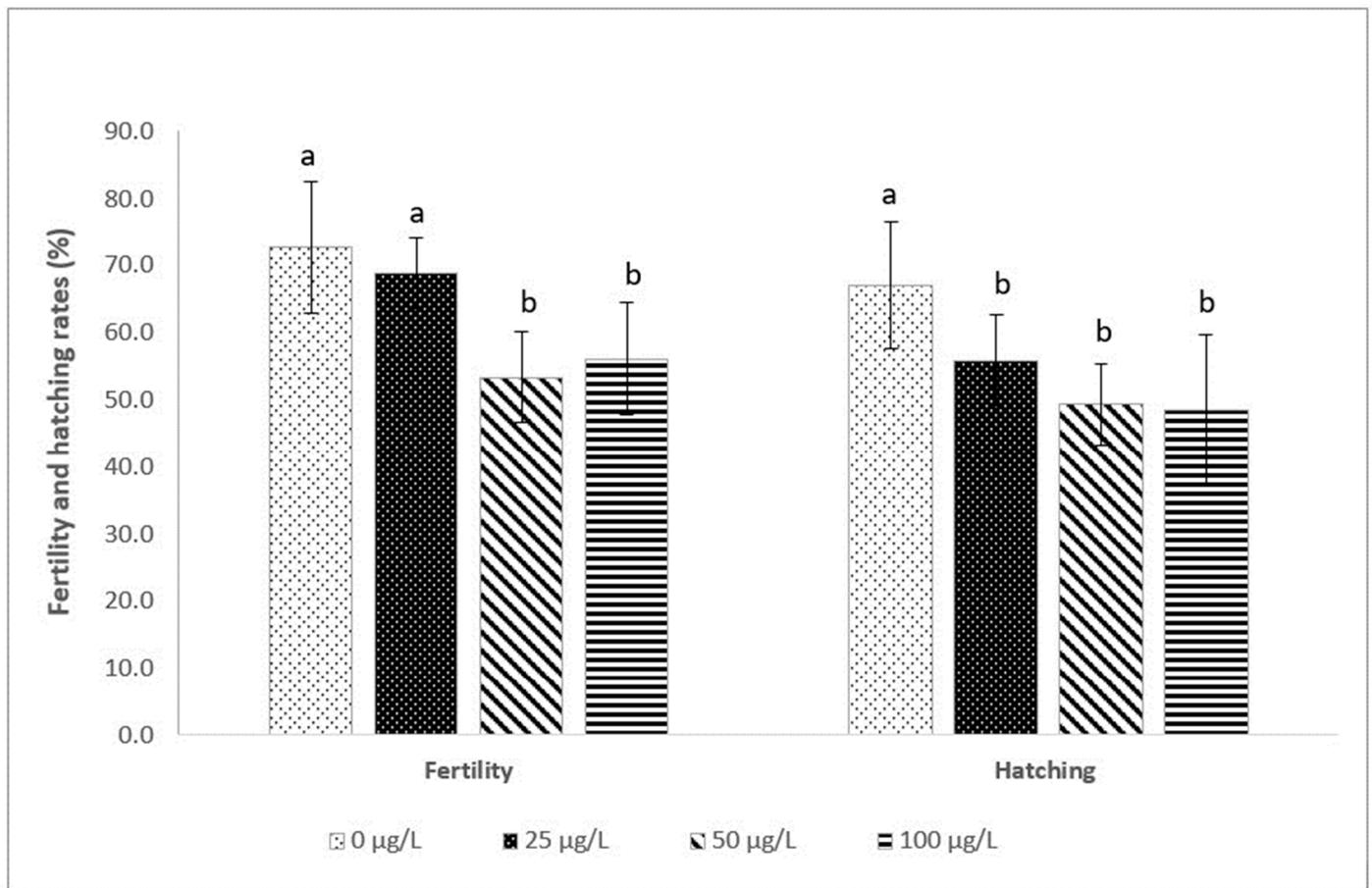
**Table 1.** The sperm quality of bocachico *Prochilodus magdalenae* activated with water contaminated with different concentrations of inorganic mercury (iHg). VCL, curvilinear velocity; VSL, straight linear velocity; STR, straightness index; LIN, linearity index; WOB, wobbling index; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Parameter (Unit)	0 $\mu\text{g/L}$	25 $\mu\text{g/L}$	50 $\mu\text{g/L}$	100 $\mu\text{g/L}$
Duration of motility (s)	35.4 $\pm$ 3.0 <sup>a</sup>	32.4 $\pm$ 2.0 <sup>b</sup>	31.0 $\pm$ 2.5 <sup>b</sup>	30.2 $\pm$ 2.3 <sup>b</sup>
Total motility (%)	94.8 $\pm$ 3.3 <sup>a</sup>	82.3 $\pm$ 9.2 <sup>b</sup>	85.5 $\pm$ 9.2 <sup>b</sup>	82.2 $\pm$ 10.3 <sup>b</sup>
Rapid (%)	39.2 $\pm$ 11.5 <sup>a</sup>	20.3 $\pm$ 10.4 <sup>b</sup>	24.3 $\pm$ 8.8 <sup>b</sup>	24.5 $\pm$ 8.1 <sup>b</sup>
Medium (%)	31.9 $\pm$ 6.5 <sup>a</sup>	23.9 $\pm$ 4.9 <sup>b</sup>	23.8 $\pm$ 4.1 <sup>b</sup>	23.6 $\pm$ 4.1 <sup>b</sup>
Slow (%)	23.8 $\pm$ 7.4 <sup>b</sup>	38.1 $\pm$ 11.6 <sup>a</sup>	37.5 $\pm$ 4.7 <sup>a</sup>	35.5 $\pm$ 6.7 <sup>a</sup>
Immotile (%)	5.2 $\pm$ 2.3 <sup>b</sup>	17.7 $\pm$ 9.2 <sup>a</sup>	14.5 $\pm$ 9.2 <sup>a</sup>	17.8 $\pm$ 10.3 <sup>a</sup>
Total progressivity (%)	57.3 $\pm$ 11.3 <sup>a</sup>	32.9 $\pm$ 13.8 <sup>b</sup>	35.1 $\pm$ 10.7 <sup>b</sup>	36.1 $\pm$ 11.0 <sup>b</sup>
VCL ( $\mu\text{m/s}$ )	92.7 $\pm$ 15.0 <sup>a</sup>	68.4 $\pm$ 16.8 <sup>b</sup>	74.0 $\pm$ 12.9 <sup>b</sup>	75.9 $\pm$ 10.6 <sup>b</sup>
VSL ( $\mu\text{m/s}$ )	46.5 $\pm$ 8.6 <sup>a</sup>	37.8 $\pm$ 12.5 <sup>a</sup>	39.4 $\pm$ 8.3 <sup>a</sup>	44.8 $\pm$ 13.2 <sup>a</sup>
STR (%)	62.2 $\pm$ 4.7 <sup>a</sup>	64.2 $\pm$ 6.1 <sup>a</sup>	62.5 $\pm$ 5.1 <sup>a</sup>	63.3 $\pm$ 4.0 <sup>a</sup>
LIN (%)	52.6 $\pm$ 4.7 <sup>a</sup>	50.5 $\pm$ 9.0 <sup>a</sup>	49.1 $\pm$ 6.2 <sup>a</sup>	51.1 $\pm$ 6.0 <sup>a</sup>
WOB (%)	82.0 $\pm$ 4.8 <sup>a</sup>	73.6 $\pm$ 8.3 <sup>b</sup>	73.8 $\pm$ 5.0 <sup>b</sup>	75.3 $\pm$ 5.4 <sup>b</sup>
ALH ( $\mu\text{m}$ )	1.6 $\pm$ 0.1 <sup>a</sup>	1.3 $\pm$ 0.1 <sup>b</sup>	1.4 $\pm$ 0.2 <sup>a</sup>	1.4 $\pm$ 0.1 <sup>b</sup>
BCF (Hz)	10.4 $\pm$ 0.6 <sup>a</sup>	8.5 $\pm$ 1.4 <sup>b</sup>	8.7 $\pm$ 1.2 <sup>b</sup>	8.9 $\pm$ 1.0 <sup>b</sup>

Different letters indicate significant differences between columns ( $p < 0.05$ ).

#### 3.2. Fertilization and Hatching Rates

The highest fertilization rates were recorded in the control (72.6  $\pm$  9.8%) and 25  $\mu\text{g/L}$  (68.8  $\pm$  5.3%) groups, with no significant difference between these values ( $p > 0.05$ ). The lowest fertilization rates were obtained in contaminated water at doses of 50  $\mu\text{g/L}$  (53.4  $\pm$  6.7%) and 100  $\mu\text{g/L}$  (56.1  $\pm$  8.4%), without observing a significant difference between these values ( $p > 0.05$ ). Also, the highest percentage of hatching was recorded in the control group (67.1  $\pm$  9.4%), with a significant difference ( $p < 0.05$ ) from the percentages obtained when the water was contaminated with iHg. Hatching rates in the treatments with iHg-contaminated water ranged between 48.5  $\pm$  11.2% (100  $\mu\text{g/L}$ ) and 55.8  $\pm$  6.8% (25  $\mu\text{g/L}$ ), without a statistical difference between these values ( $p > 0.05$ ) (Figure 1).



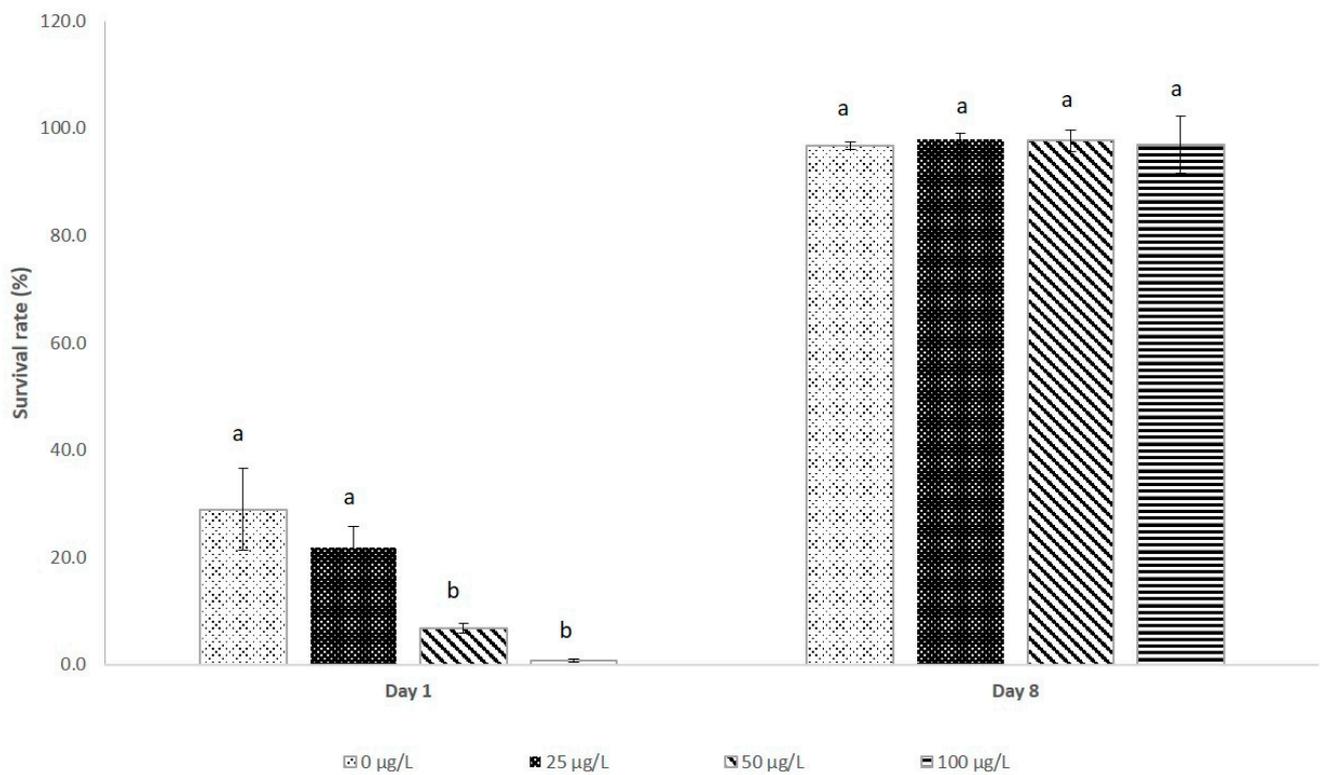
**Figure 1.** Fertilization and hatching rates of bocachico *Prochilodus magdalenae* were obtained with gametes activated and incubated during the first hour with water contaminated with different inorganic mercury (iHg) concentrations. Different letters indicate significant differences ( $p < 0.05$ ).

### 3.3. Larval Survival

At 1 dph, the lowest survival rates were recorded in the treatments with contaminated water at 50 µg/L (6.8%) and 100 µg/L (0.8%), with no significant difference between these values ( $p > 0.05$ ). The highest survival was recorded in the control group (29.0%) and in gametes treated with 25 µg/L (21.8%) ( $p > 0.05$ ). At 8 dph, survival oscillated between 96.8% (control) and 98.1% (25 µg/L), with no significant difference between these treatments ( $p > 0.05$ ) (Figure 2).

### 3.4. Egg Diameters, Mercury Absorption by Eggs and Larvae, and Size of Newly Hatched Larvae

The diameter of the oocytes was greatest in the treatments with contaminated water at 50 µg/L ( $4.9 \pm 0.4$  mm) and 100 µg/L ( $4.9 \pm 0.5$  mm) iHg, with a statistical difference ( $p < 0.05$ ) from oocyte diameter in the control group ( $4.3 \pm 0.3$  mm) and the 25 µg/L group ( $4.5 \pm 0.3$  mm). Also, dose-dependent uptake of iHg was observed in eggs and newly hatched larvae. The higher the concentration of iHg in the water, the higher the absorption by embryos and larvae. However, despite the changes in the diameter of the oocytes, the size of the newly hatched larvae (day 1) did not show significant differences between the different treatments ( $p > 0.05$ ) (Table 2).



**Figure 2.** The larval survival rate of bocachico *Prochilodus magdalenae* on the first and eighth day post-hatching. The larvae were obtained using gametes activated and treated during the first hour of incubation with water contaminated at different inorganic mercury (iHg) concentrations. Different letters indicate significant differences ( $p < 0.05$ ).

**Table 2.** Egg diameters, inorganic mercury absorption by eggs and larvae, and total length of newly hatched bocachico *Prochilodus magdalenae* larvae (1 day old). The larvae were obtained with gametes activated and incubated through the first hour with water contaminated at different inorganic mercury concentrations. tL, total length.

Parameters	0 µg/L	25 µg/L	50 µg/L	100 µg/L
Egg diameters (mm)	4.3 ± 0.3 <sup>a</sup>	4.5 ± 0.3 <sup>b</sup>	4.9 ± 0.4 <sup>c</sup>	4.9 ± 0.5 <sup>c</sup>
iHg absorption by eggs (µg/kg of eggs)	1.1 ± 0.2 <sup>c</sup>	64.5 ± 3.5 <sup>b</sup>	447.6 ± 367.9 <sup>b</sup>	1603.3 ± 766.1 <sup>a</sup>
tL of day-old larvae (mm)	8.2 ± 1.2 <sup>a</sup>	8.3 ± 1.2 <sup>a</sup>	8.0 ± 0.9 <sup>a</sup>	8.3 ± 0.6 <sup>a</sup>
iHg absorption by day-old larvae (µg/kg of larvae)	115.4 ± 4.6 <sup>c</sup>	150.7 ± 10.7 <sup>b</sup>	192.35 ± 11.10 <sup>a</sup>	214.8 ± 21.2 <sup>a</sup>

Different letters indicate significant differences between columns ( $p < 0.05$ ).

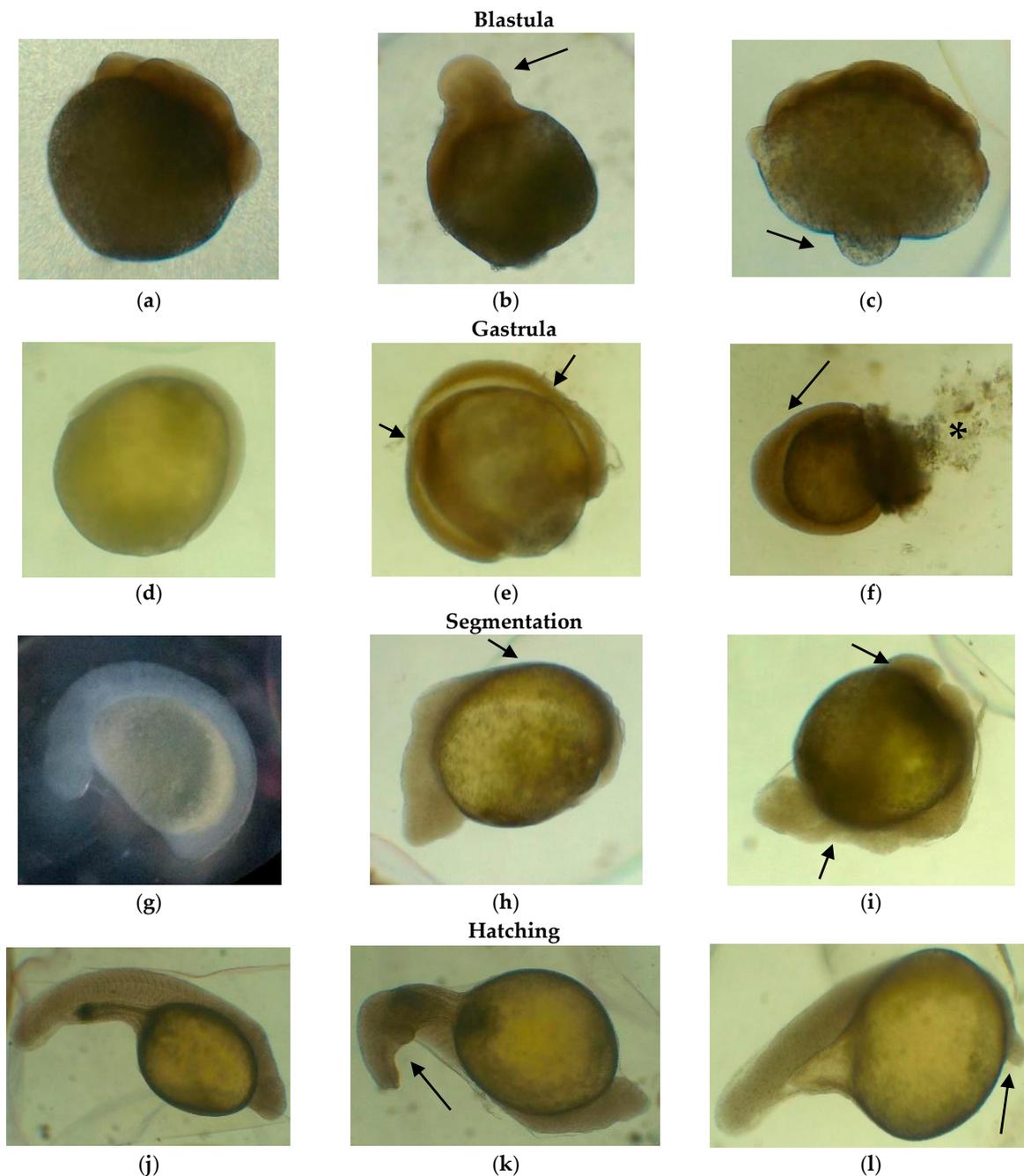
### 3.5. Deformation of Embryos and Larvae Exposed to Inorganic Mercury

In the treatments with water contaminated with iHg, the embryos' deformations were significantly greater than those in the control group ( $p < 0.05$ ). The deformations occurred in all stages of embryonic development and became more evident as embryo development advanced. At the pharyngula stage, the embryo deformation rates were significantly higher in all iHg-treated groups than in the control (Table 3, Figure 3).

**Table 3.** Deformation percentages at different stages of the embryonic development of bocachico *Prochilodus magdalenae*. The embryos were obtained with gametes activated and incubated through the first hour with water contaminated with different inorganic mercury concentrations. HPF, hours post-fertilization.

Hours (Stage)	0 µg/L	25 µg/L	50 µg/L	100 µg/L
1 HPF (blastula)	4.7 ± 1.2 <sup>c</sup>	7.4 ± 2.8 <sup>b</sup>	8.8 ± 4.0 <sup>ab</sup>	17.6 ± 5.0 <sup>a</sup>
5 HPF (gastrula)	4.8 ± 3.3 <sup>c</sup>	7.0 ± 2.6 <sup>bc</sup>	15.3 ± 6.2 <sup>a</sup>	11.2 ± 8.8 <sup>ab</sup>
8 HPF (segmentation)	8.2 ± 2.9 <sup>b</sup>	15.6 ± 5.2 <sup>a</sup>	19.1 ± 5.3 <sup>a</sup>	15.0 ± 1.9 <sup>a</sup>
11 HPF (pharyngula)	10.0 ± 7.7 <sup>b</sup>	45.8 ± 9.1 <sup>a</sup>	49.3 ± 16.0 <sup>a</sup>	46.6 ± 9.2 <sup>a</sup>

Different letters indicate significant differences between columns ( $p < 0.05$ ).



**Figure 3.** Microphotographs of deformed embryos of the bocachico *Prochilodus magdalenae*. The gametes were activated and incubated with water contaminated with inorganic mercury (iHg) through

the first hour. (a) Normal embryo in the cleavage stage; (b) embryo with anomalies in the blastomeres; (c) embryo with yolk deformation; (d) normal embryo in the gastrula stage; (e) embryo with deformation of the blastoderm; (f) embryo with deformation of the blastoderm and detachment of cellular material; (g) normal embryo in the segmentation phase; (h) embryo with deformation in the cephalic region; (i) embryo with deformation in the cephalic and caudal regions; (j) normal hatching embryo; (k) embryo with deformation in the dorsal spine (lordosis); (l) embryo with head deformation (microcephaly). The arrow indicates the injury.

#### 4. Discussion

The results of trial 1 showed a decrease in the semen quality of bocachico when milt was exposed to water contaminated with iHg. Parameters such as total motility (9.8–13.2%), duration of motility (8.5–14.7%), WOB (8.2–10.2%), ALH (12.5–18.8%), BCF (14.4–18.3%), VCL (18–26%), and rapid motility (38–48.2%) decreased when milt was activated with water contaminated with iHg at a dosage between 25 to 100 µg/L; in contrast, the quantity of immotile sperm increased 3-fold relative to that in the control (Table 1).

In studies carried out with other species of fish, alterations have been reported when milt is activated with water contaminated with inorganic mercury. Hayati et al. [51] reported a 20% decrease in motility duration of sperm from the common carp *Cyprinus carpio* when they activated semen with mercuric chloride contaminated water at dosages between 1000 and 5000 µg/L. Dietrich et al. [33] also reported decreased total motility below 50% and a significant reduction of VSL but not VCL in the semen of rainbow trout *Oncorhynchus mykiss* when they activated the sperm with inorganic-mercury-contaminated water ( $\text{Hg}^{2+}$ , iHg) at 10,000 µg/L. Pataki et al. [52] suggested that the total motility and VCL of zebrafish *Danio rerio* semen decreased when the sperm was activated with Hg-contaminated water between 500 and 5000 µg/L; however, they found that with older fish (7, 12, and 18 months) these parameters suffered more remarkable alterations. In the present study, the semen evaluated was extracted from bocachico breeders of the same age (24 months). It has been reported that semen tolerance to mercury contamination is species-specific and depends on the characteristics of the spermatozoa and the physiology and biochemistry of the seminal plasma of each species [32,53]. Therefore, considering the results of this study and those obtained for other fish species, we can suggest that bocachico semen is sensitive to mercury contamination (iHg) from a concentration of 25 µg/L.

In general, a decrease in sperm kinetics suggests a disruption in energy metabolism and/or damage to the mitochondria [32,54,55]. Mercury in its different forms (organic and inorganic) in high concentrations induces cytotoxicity mediated by reactive oxygen species (ROS) and could be toxic to the male reproductive system of fish, affecting testicular spermatogenic and steroidogenic functions [6,56,57]. In addition, Hg has been shown to delay spermatogenesis through inhibitory actions of pituitary gonadotropins, decreases in the gonadosomatic index, or inhibition of the spermatid-to-sperm transformation [6,25].

The results of trial 2 showed that the lowest fertilization and hatching rates were obtained at the highest doses of iHg (50 µg/L and 100 µg/L). These treatments showed a significant decrease in fertilization (23–26%) and hatching (27–28%) rates relative to those in the control group. In the common carp *Cyprinus carpio*, a decreased fertilization rate from exposure to 500 µg/L Hg was reported [51]. In the rainbow trout *Oncorhynchus mykiss*, when eggs were exposed to Hg at 10,000 µg/L for four hours, the hatching rate was reduced to one-third of that obtained in the group control [26]. These results suggest that the inorganic mercury intoxication of bocachico gametes and embryos, particularly in the permeability stage of the chorion, reduces fertilization and hatching rates at concentrations above 50 µg/L, which indicates a greater sensitivity of this species to the effects of mercury.

The early period of embryo development just after fertilization and the hatching period are the most sensitive to metal poisoning. Alterations during these periods reduce the quantity and quality of larvae [26]. The decrease in fertilization capacity due to exposure to contaminants is not only related to the inhibition or suppression of the onset of motility

and sperm velocity due to a decrease in ATP content but also to damage to DNA integrity due to oxidative stress produced by reactive oxygen species (ROS) [32,58–60].

In the present study, the percentage of embryo deformations was higher in the treatments exposed to iHg than in the control group. Although deformations were observed in all stages of embryonic development, the abnormalities were more expressed as development progressed. At the pharyngula stage, the embryonic deformation rate in iHg-treated groups was four times higher than that estimated in the control group. The malformations were expressed in different parts of the embryo (blastomeres, blastoderm, yolk).

In the blastomeration state, abnormalities in blastomere distribution, asymmetry in size, and disturbances during cell division were observed; yolk alterations were also observed. In the gastrulation state, deformations were observed in the blastoderm, and detachment of the cellular material was noted. In the segmentation stage, deformations were observed in the caudal and cephalic regions. In the pharyngulation stage, malformations were observed in the head (microcephaly) and the spinal column (scoliosis or lordosis). Deformation percentages were highest in the final stage of embryo development (pharyngulation). In common carp, egg agglomerations and embryo malformations were reported after inorganic mercury exposure (12 and 24 h) at much higher doses (500 µg/L) than in the present study. These damages were attributed to the toxic nature of mercury and the increase in reactive oxygen species (ROS), which affected fish fertility and embryo development [51].

Exposure of bocachico eggs to different iHg concentrations (25, 50, and 100 µg/L) during the first hour of hydration caused damage to the embryos. In the oocytes exposed to different iHg concentrations, an increase in diameter was recorded relative to that the control group. The diameter of the eggs increased significantly, with values between 4.7% (25 µg/L) and 14.0% (50 and 100 µg/L) greater than control values.

Moreover, eggs absorbed more inorganic mercury at higher heavy metal concentrations in the water; this suggests that mercury absorption by eggs depends on the metal concentration in the water. The oocytes exposed to 100 µg/L absorbed 16 times the concentration to which they were treated ( $1603.3 \pm 766.1$  µg/kg); eggs subjected to 50 µg/L absorbed approximately nine times the dose to which they were exposed ( $447.6 \pm 367.9$  µg/kg), and the eggs exposed to 25 µg/L absorbed about 2.5 times the exposed concentration ( $64.5 \pm 3.5$  µg/kg). The oocytes of the control treatment (0 µg/L) presented an iHg concentration of  $1.1 \pm 0.2$  µg/kg ( $p < 0.05$ ). The iHg concentrations in the eggs of the control group may be attributed to the volatility of mercury, which may have been distributed throughout the experimental trial environment, despite the measures taken to prevent this effect.

The increase in the diameter of the eggs could be attributed to the absorption of iHg, which caused a high osmolarity and, therefore, a high intra-oocyte osmotic pressure that, in some cases, caused premature hatching or abortion in the contaminated treatments at the highest iHg concentrations (50 and 100 µg/kg). Nevertheless, it was also observed that the deformations impeded the movements of the embryos required to break the chorion before hatching (weak embryos), especially in the treatments contaminated with the highest doses of iHg evaluated in the present study (50 and 100 µg/L). Likewise, these treatments showed high deformations in the dead larvae on the first day post-hatching (dph). It should be noted that the larvae that survived after 1 dph were resistant, without evident malformations. Mortality was low between 1 and 8 dph, and survival in that period was similar between the different treatments.

It is convenient to note that more research is necessary to understand the chemical interactions of chorion components with different xenobiotics and the ability of contaminants to cross the chorion. It is necessary to deepen the knowledge of the composition and dynamics of the chorion pores (permeability), the only route of hydration for the eggs. It is known that the permeability of the chorion varies during development and is affected by environmental conditions. However, there is no clarity about the types of molecules for which the chorion is permeable or acts as a barrier [36,61]. Therefore, more studies on the

permeability of the chorion of the eggs of migratory fish such as bocachico is recommended, allowing for more precise identification of the causes of embryonic and larval deformations and the high mortality caused by heavy metals.

## 5. Conclusions

The results of the present study reveal that during the reproduction of bocachico in contaminated waters, inorganic mercury affects sperm quality and embryonic development from 25 µg/L, as well as fertilization and hatching rates from 50 µg/L. In addition, the results suggest that bocachico *Prochilodus magdalenae* is highly sensitive to the toxic effects of mercury in the early stages of its life cycle.

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**Institutional Review Board Statement:** The study was conducted in accordance with the Rules of Conduct for the Use of Animals in Teaching and Research of the Faculty of Veterinary Medicine and Zootechnics of the University of Córdoba (Colombia) and approved by the Ethics Committee (Act 009 of 30 November 2021).

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