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Research Article

Effects of Sublethal Concentrations of the Herbicide, Glyphosate, on Embryonic Development of the Indian Major Carp, *Labeo rohita*

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Aquaculture is one of the fastest growing sectors worldwide. Currently, 50% of global fish consumption is provided by aquaculture. In India, the major cultivated fishes are *Labeo catla, Labeo rohita, Cirrhinus mrigala, Hypophthalmichthys molitrix, Ctenopharyngodon idella*, and *Cyprinus carpio*. Among these, *L. rohita* is a widely consumed fish. We aimed to study the effect of one of the most widely used herbicides, glyphosate, on the embryonic development of *L. rohita.* The 96 h LC_{50} of glyphosate for *L. rohita* embryos was found to be 20.89 mgL^{-1}. The embryos were exposed to $1/10^{th}$, $1/5^{th}$, and $1/3^{rd}$ concentrations of LC_{50} for 96 h. The observed deformities included abdominal curvature, kink formation in the tail, yolk sac edema, pericardial edema, and improperly flattened swim bladder. Besides, we could observe a reduction in pigmentation at 96 h and a decrease in heart rate at 24, 48, 72, and 96 h. All these deformities led to the mortality of embryos. This study indicated that the herbicide (glyphosate) can adversely affect the natural population of the Indian major carp, *L. rohita*.

1. Introduction

Glyphosate [*N*-(phosphonomethyl) glycine], CAS Number: 1071-83-6, is a chemical used for weed control. The half-life period of the molecule ranges from 2 to 215 days in soil and 2 to 91 days in water [1]. Weeds compete with cultivated crops for nutrients and sunshine. They adversely affect the growth of plants [2]. Glyphosate has inhibitory effects on EPSPS (5-enolpyruvylshikimate 3-phosphate synthase). EPSP inhibition leads to the depletion of the aromatic amino acids tryptophan, tyrosine, and phenylalanine that are needed for protein synthesis. This organophosphate group of pesticide was first synthesized and commercialized in 1950 by a pharmaceutical company of Switzerland, but its herbicidal properties were unknown. E. Franz of Monsanto Company studied the herbicidal properties of glyphosate under the trade name Roundup [3]. Since then, it has been used in agriculture, horticulture, and forestry [4]. Its use became widespread after the discovery of glyphosate-resistant crops in 1980, and it has been increased by 100 fold; till date, it is the most abundantly used herbicide worldwide [5]. This polyprotic molecule has phosphonate, carboxyl, and amino groups as three polar functional groups [6]. Glyphosate has been used as a herbicide for many years [5, 7]. But during the last few years, it has been banned in some countries worldwide, since it affects soil, water, and soil microbiota. In India, it has been banned in Kerala and Punjab. In other states, it is permitted for restricted use only. In Odisha,

glyphosate is commonly used for weed management in rice, maize, cotton, and vegetable cultivations [8–10]. Glyphosate (GLY) has been classified as a Group 2B and Group 2A carcinogens for humans [11, 12], respectively, and frequently found in aquatic ecosystems. To date, GLY has been detected in natural waters at concentrations generally between 3 and 700 μg/L in many countries [13].

Aquaculture is the controlled process of breeding, raising, and harvesting finfish, shellfish, and aquatic plants for human consumption. Fish gives hope for fighting malnutrition since it is a food that is rich in protein, calcium, omega-3 fatty acids, and vitamins like A and B_{12} [14]. Currently, 50% of global fish consumption is fulfilled by aquaculture [15]. Besides, it creates scope for employment and contributes to economic growth. Fish and aquaculture support the livelihood of 12% of the global population. It is one of the fastest-growing sectors worldwide.

According to the State of World Fisheries and Aquaculture study, the growth of aquaculture, particularly in Asia, has raised the total production of fisheries and aquaculture to 214 million tons in 2020 comprising 178 million tons of aquatic animals and 36 million tons of algae [16]. Globally, India is the second largest producer of fish after China. Around 95% of total aquaculture production is contributed by freshwater aquaculture. The application of advanced technologies has resulted in an increase in aquaculture production in India by 7%, with an annual production of 5.77 million tons. This upward shifting of the growth curve has been achieved by induced breeding of fishes like the three Indian major carps (*Labeo catla, Labeo rohita*, and *Cirrhinus mrigala*) and composite culture of major carps as well as exotic carps (*Hypophthalmichthys molitrix*, *Ctenopharyngodon idella,* and *Cyprinus carpio*) [8]. Out of these, *L. rohita* is a highly consumed farmed fish. The presence of pollutants in water bodies affects the growth and development of fish and ultimately adversely affects the aquaculture output in terms of quality and quantity. The development of fish is dependent upon the factors present in the surrounding environment. So, any alteration in surroundings can induce developmental changes and these changes may persist to adulthood, even if exposure to pollutants is ceased. This study aims to determine the developmental toxicity of one of the widely used herbicides, glyphosate, on the commercially important fish, *L. rohita*.

2. Materials and Methods

2.1. Test Chemicals. The commercial grade of glyphosate-based herbicide, "Clear off," was obtained from Safex Chemicals Ltd., Delhi, India. The effective concentration was 41%. The three different concentrations taken were $1/3^{rd}$, $1/5^{th}$, and $1/10^{th}$ of LC_{50} (6.96 mgL^{-1}, 4.18 mgL^{-1}, and 2.08 mgL^{-1}, respectively). To prepare these concentrations in 500 ml of water, 8.7, 5.22, and 2.6 μl were mixed into water, respectively.

2.2. Collection of Eggs. Fertilized eggs of *L. rohita* were collected from ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, Carp Hatchery Unit. A pair of 3-year-old male and female fish was bred. The female fish weighed 2.2 kg with 38.5 cm length. The male fish was of 1.8 kg weight with 33 cm length.

2.3. Screening of Fertilized Eggs. The fertilized eggs were screened in the laboratory depending on their transparent appearance which was visible by naked eyes. The unfertilized dead embryos were opaque in appearance. The embryos were observed under microscope and those showing cleavage were selected for the toxicity test.

2.4. Median Lethal Concentration. The median lethal concentration (LC_{50}) was determined by Finney's method [17]. To prepare 1, 10, 20, 30, 40, and 50 mgL^{-1} concentrations of glyphosate, 0.25, 2.5, 5.0, 7.5, 10, and 12.5 μl volumes were added in 80 ml of water, respectively. Ten fertilized eggs were released to each concentration 4 h after fertilization. The number of dead embryos was counted and noted down at an interval of 24 h, and the rate of mortality was calculated. Then, the probit value was noted down from Finney's table. The logarithm values of respective concentrations were calculated. A graph was plotted taking logarithm values of concentrations along the *X*-axis and probit values of mortality in respective concentrations along the *Y*-axis. Then, probit of 5 was located on *Y*-axis. Then, by moving down the *X*-axis, we could find the logarithm of $LC_{50.}$ By calculating the anti-logarithm, we could determine the LC_{50}.

2.5. Exposure of Embryos to Glyphosate. The 4 hpf (hours post fertilization) embryos were exposed to $1/3^{rd}$, $1/5^{th}$, and $1/10^{th}$ of the median lethal concentration of glyphosate. The three treatments were designated as treatment-I, II, and III depending upon the concentration of glyphosate. The dosages were 2.08, 4.17, and 6.96 mgL^{-1} for treatment-I, treatment-II, and treatment- III, respectively. The concentrations were loaded 30 min before the release of embryos. Fifty embryos were released to each Petri plate containing 500 ml of water. The experiment was conducted in a set of 3 replicates. The embryos were observed under a microscope and photographed. The observations include duration of hatching, heart, and optic cup formation, eye development, formation of the branchial artery, edema, and kink formation. The percentage of deformed embryos was calculated for 24, 48, 72, and 96 h as a function of time. The percentage of deformed embryos was calculated as the ratio of deformed embryos/larvae to the number of alive embryos/larvae at 24 h. For 48, 72, and 96 h, it was calculated as a cumulative percentage.

3. Results

The median lethal concentrations for 72 and 96 h were 26.3 and 20.89 mgL^{-1} (Figure 1), respectively. The differentiation of the head, yolk sac, and tail was observed in all treatments and control at 13 hpf. The twitching movement was also observed after the 13^{th} hour of development. In control and treatment-I, hatching occurred between the 16^{th} and 27^{th} hpf, whereas in treatment-II and treatment-III, it occurred

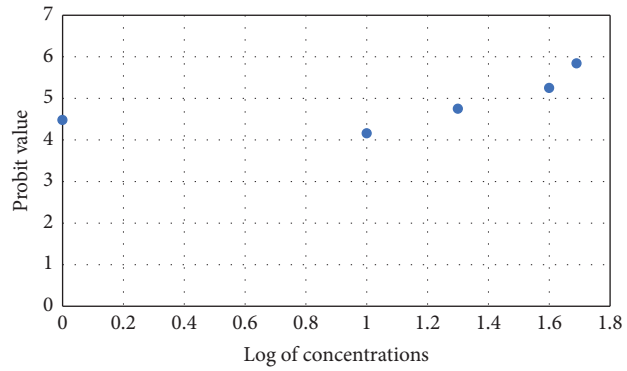

FIGURE 1: The graph showing the 96 h LC_{50} value of glyphosate for embryos of *Labeo rohita*.

between the 16th and 22nd hpf. The optic cup formation was delayed in all three treatments. In control, it occurred at 24 hpf, but in all three treatments, it was found at 27 hpf. At the 16th hour of development, the red blood cells (RBCs) were formed in all treatments and control except two embryos in treatment-II. The branchial arteries were formed at 37 hpf in control and all treatments. Other deformities observed were yolk sac edema, pericardial edema, abdominal curvature, and kink formation in the tail. At 24 h, 2 deformed embryos were observed in treatment-I with yolk sac edema, pericardial edema, and elongated yolk sac. In the rest of the treatments and control, all embryos were normal (Figure2). At 48 h, more abnormalities were observed at higher concentrations as compared to lower concentrations (Figure 3). At 2.08 mgL^{-1}, 4 deformed embryos were found, of which 3 contained yolk sac edema and 2 had pericardial edema. In treatment-II, 7 deformed embryos were observed, of which 4 had deformed yolk sacs and pericardium, and 6 embryos contained deformed spinal cords. Eight deformed embryos were observed in treatment-III, out of which 4 embryos had yolk sac and pericardial edema and 2 were with kink formation in the tail. At 72 h, 6 embryos were detected with yolk sac edema, 4 with pericardial, 1 with abdominal curvature, and 2 with tail kink (Figure 4). In treatment-II, 6 embryos were having yolk sac edema, 4 with pericardial edema, and 3 with kink formation in the tail. In the highest concentration of glyphosate, yolk sac edema, pericardial edema, and abdominal curvature were noticed in 6, 5, and 1 embryos, respectively. At 96 h, more changes were observed in the high concentration as compared to the lower concentrations (Figure 5). The tail kink formation was observed with 1 embryo in both treatment-I and treatment-II. In treatment-III, abdominal curvature was observed in one embryo. The embryos treated with glyphosate showed less pigmentation. Besides, the air bladder was not properly flattened in the embryos of treatment-III. The percentage of deformed embryos in various concentrations of glyphosate at different time intervals is mentioned in Table 1. The heart rate was calculated in embryos of all the treatments and control at an interval of 24 h (Figure 6). It was found that in all four observations, the heart rate of control embryos was higher than treatments and it decreased in a concentration-dependent manner.

4. Discussion

In this experiment, we studied the sublethal effects of glyphosate on the development of embryos of *L. rohita*. The 96 hpf is a crucial period for embryos. In zebrafish, more developmental abnormalities were observed when the embryos were exposed to glyphosate for 96 h after 4 h of fertilization as compared to the exposure for 96 h at 3 days post fertilization [18]. Hence, in this experiment, we exposed the 4 hpf embryos to different concentrations of glyphosate for a period of 96 h. It could be observed that the deformities in embryos increased with an increase in glyphosate concentration and duration of exposure. The same observations were reported in zebrafish exposed to 1, 5, 10, and 100 mg/L concentrations of glyphosate for 24, 48, 72, and 96 h [19]. Before hatching, no deformities were observed in any treatment or control due to the presence of chorion membrane. The chorion is a secondary membrane secreted by the developing oocyte. It is a cellular layer present immediately above the plasma membrane of an ovum [3, 20]. This membrane protects the embryo from pollutants present in the external environment. According to the study by Villalobos et al. [21], the chorion membrane protects the medaka embryos from the toxic effects of Thiobencarb. This information supports our findings.

The heart rate was found to be decreased in all the treatments compared to the control. It slowed down with an increased concentration of glyphosate. In the case of medaka embryos, the heart rate increased at first and then it decreased. The increased heart rate was described as an adaptation to stress environment, and the decreased heart rate might have resulted due to destruction of cells in the heart wall [22]. Our findings differed from it. The heart rate was decreased in glyphosate-treated fish in a dose-dependent manner. The same results were observed in the case of zebrafish exposed to 50 μg/ml of glyphosate. They described it as a result of cell death in the heart and the concentrations taken were higher, so the embryos could not acclimate to the stress condition [23].

The first deformities were observed in 2.08 mgL^{-1} concentration at 23rd h. We could observe 2 deformed embryos in the 23rd h of development at 2.08 mgL^{-1} only, but no deformities were observed at higher concentrations. The embryos are more susceptible to lower concentrations of glyphosate during early stages within 24 h. During later stages of development, they were more susceptible to higher concentrations (Table 1). Such changes were observed in zebrafish exposed to methyl mercury. More number of deformed embryos were observed at 20 μg/l compared to 30 μg/l after 16 hpf [24]. The spinal curvature and kink formation in the tail were observed in all the treatments, but not in the control. Hence, it is clear that these changes resulted due to exposure to glyphosate. These could be due to decreased collagen synthesis. In the case of medaka embryos, several developmental deformities were reported, such as bent tail and abdominal enlargement after exposure to glyphosate. The concentrations used in this experiment (100, 200, 300, 400, and 500 mgL^{-1}) were much higher than the concentrations used in our experiment [22]. This indicates

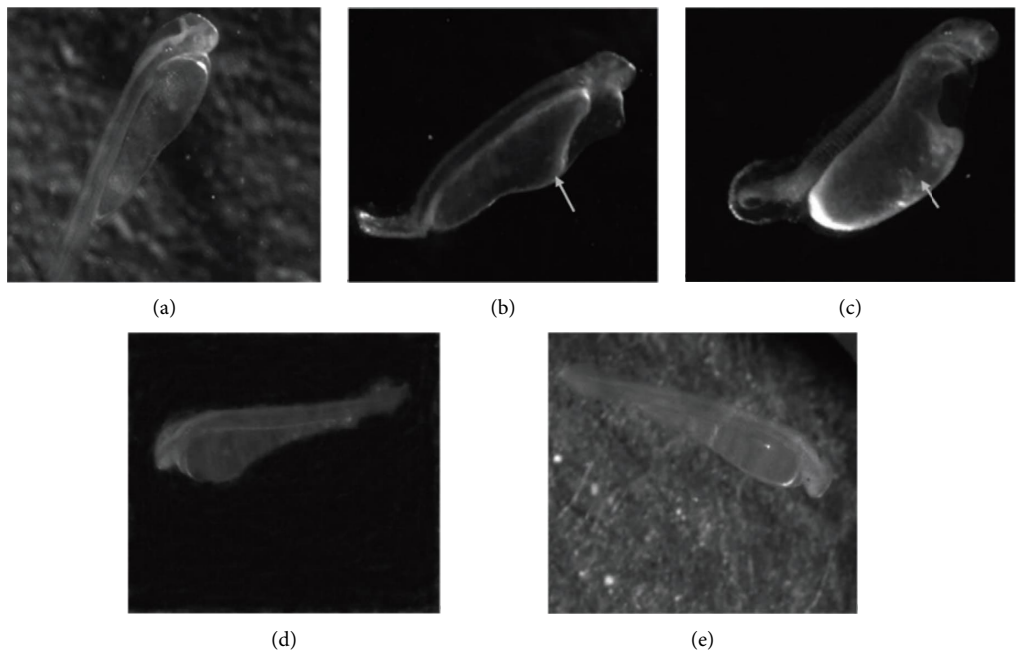

Figure 2: Development of embryos of *Labeo rohita* at 24 hpf. (a) Normal hatchling in control. (b) Kink formation in the tail and pericardial edema in T-I. (c) Elongated yolk sac and incomplete differentiation of tail in T-I. (d) Normal hatchling in T-II. (e) Normal hatchling in T-III.

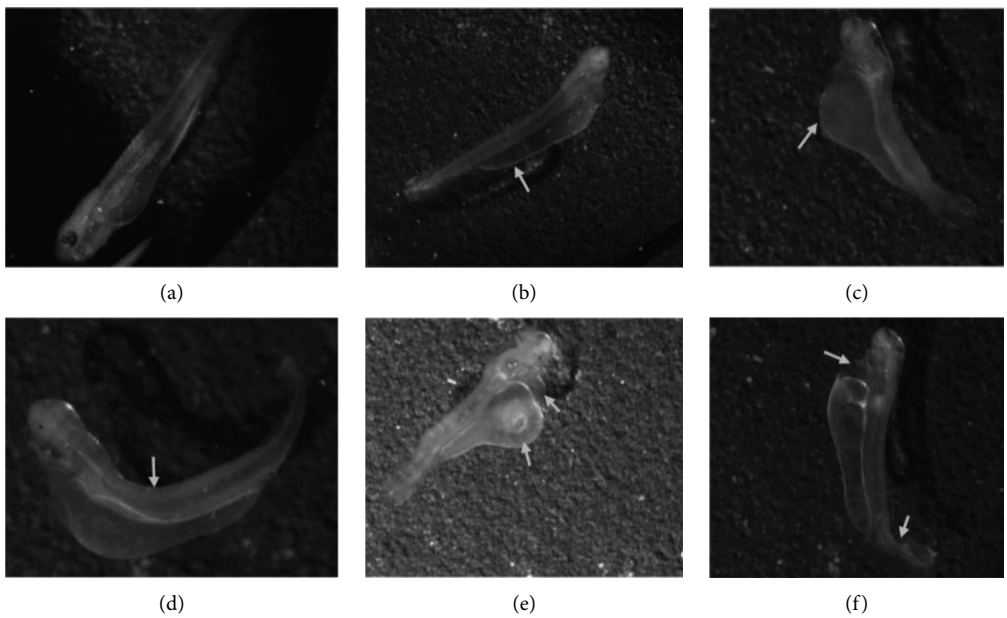

Figure 3: Developing embryo of *Labeo rohita* at 48 hpf: (a) normal developing embryo in control, (b) embryo with an elongated yolk sac in T-I, (c) yolk sac edema in an embryo of T-I, (d) abdominal curvature in an embryo of T-II, (e) yolk sac and pericardial edema in T-II embryo, and (f) pericardial edema with kink formation in the tail in T-III embryo.

that the embryos of *L. rohita* are more sensitive to glyphosate as compared to medaka.

Spinal curvature was reported in zebrafish exposed to 0.620 (0.436–0.963), 0.475 (0.302–0.801), and 0.341(0.177–0.617) $mg{\cdot}L^{-1}$ concentration of organophosphate insecticide, Sumithion [1]. Glyphosate was identified as a potent teratogen to the Japanese medaka fish (*Oryzias latipes*) embryos and can induce developmental abnormalities at a concentration of 0.5 mg/L [25]. The abnormalities include spinal curvature, enlarged yolk sac, and greying of the yolk sac. The yolk sac edema was noticed in the African catfish (*Clarias gariepinus*) exposed to a group of metallic chemical elements, such as chromium, cadmium, copper, and agrochemicals like sodium pentachlorphenol (NaPCP) and malathion [26]. The yolk sac is the site for early blood flow. Remodeling of blood vessels occurs at this site. Glyphosate

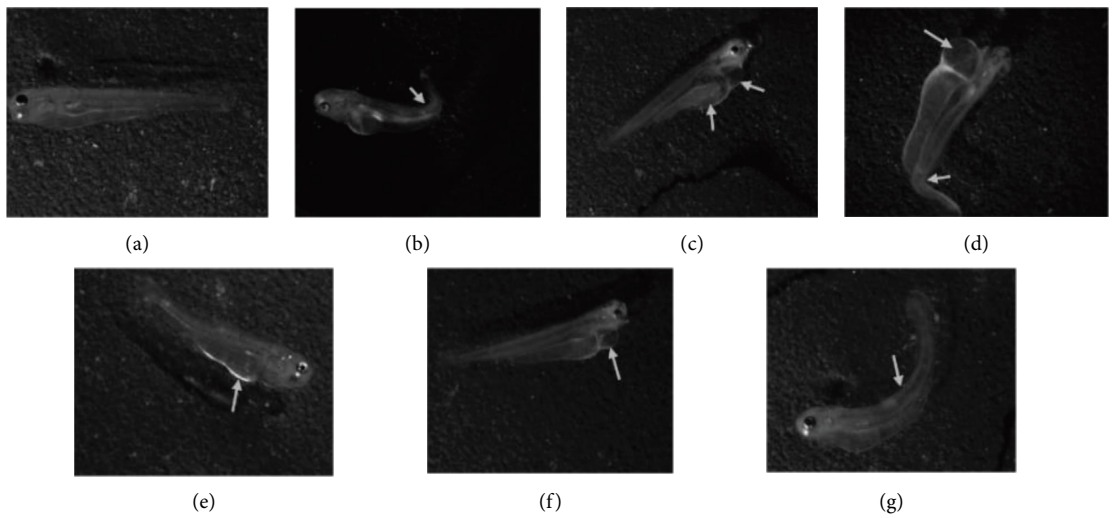

Figure 4: Developing embryo of *Labeo rohita* at 72 hpf: (a) normal developing embryo in control, (b) tail curvature in T-I embryo, (c) yolk sac and pericardial edema with an embryo of T-I, (d) yolk sac and pericardial edema with tail kink formation in T-II embryo, (e) yolk sac edema and pericardial edema in an embryo of T-II, (f) pericardial edema in T-III embryo, and (g) abdominal curvature in T-III embryo.

Table 1: The percentage of deformed embryos of *Labeo rohita* at various concentrations of glyphosate at different time intervals.

Treatments/time interval	Percentage of deformed embryos			
	24 h	48 h	72 h	96 h
Control	0	0	0	0
Treatment-I	2	11	33	46
Treatment-II	0	14	36	53
Treatment-III	0	17	42	62

Treatment-I: 2.08 mgL^{-1}, treatment-II: 4.17 mgL^{-1}, and treatment-III: 6.96 mgL^{-1}.

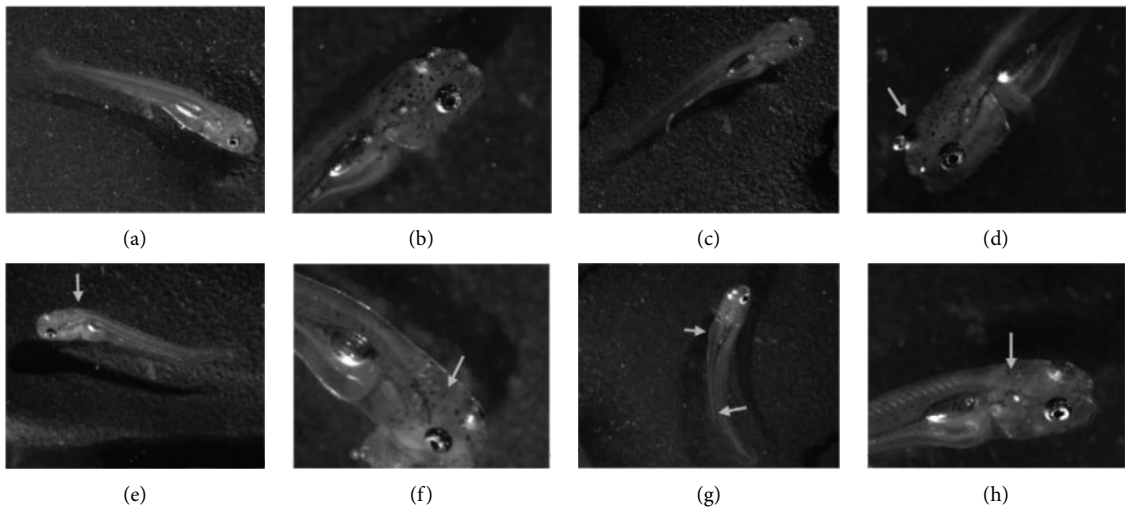

Figure 5: Developing embryo of *Labeo rohita* at 96 hpf: (a, b) normal developing embryo in control, (c, d) an embryo of T-I showing reduced pigmentation, (e) embryo with spinal deformity in T-II, (f) reduced pigmentation in T-II embryo, (g) developing embryo in T-III with abdominal curvature and improperly fattened swim bladder, and (h) reduced pigmentation in T-III embryo.

caused rupture of early blood vessels near yolk sac. The yolk sac edema was observed in zebrafish (*Danio rerio*) after 96 h of exposure to 2,3,7,8-tetrachlorodibenzo-*p*-di-Polychlorinateddibenzo-*p*-dioxins (PCDDs) and dibenzo-oxin (TCDD) [27]. In this experiment, the first yolk sac edema was observed at 24 h at a 2.08 mgL^{-1} concentration of glyphosate.

The well-developed swim bladder was observed at 96 hpf. This organ serves as a bouncy device. In teleost, it

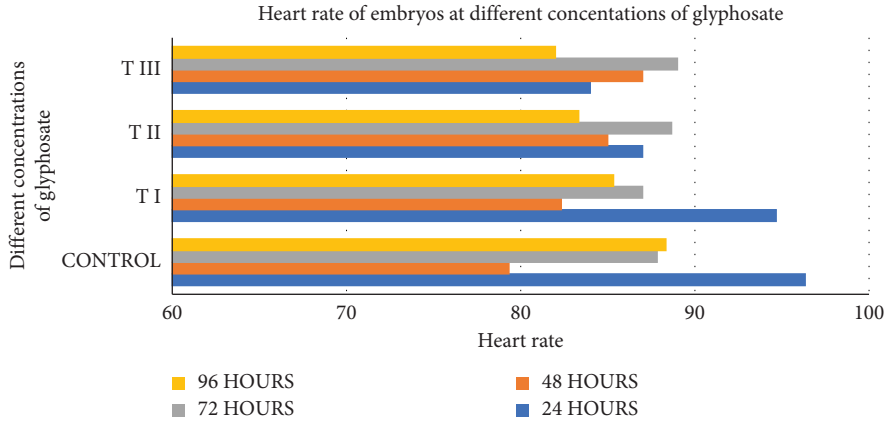

Figure 6: Heart rate of *Labeo rohita* embryos in different treatments (2.08, 4.1, and 6.96 mgL^{-1}) of glyphosate.

consists of two chambers, anterior and posterior. The posterior chamber is vascularised and gas release occurs in this chamber [28]. In the case of carp, the one-chambered swim bladder develops at 96 hpf and the two-chambered swim bladder develops at 192 hpf [29]. In our experiment, the swim bladder was improperly flattened only at a 6.96 mgL^{-1} concentration of glyphosate. A similar observation was made in zebrafish exposed to glyphosate-based herbicides. The different concentrations were 11.7, 35, and 58.3 mgL^{-1}. The abnormality was observed in a dose-dependent manner [10]. A reduced pigmentation was observed in glyphosate-treated embryos. Such a reduction in pigmentation has been observed in algae treated with various pesticides. However, no such study has been conducted on animals. The effects of glyphosate on chromatophore cells can be explored further.

5. Conclusion

This research presents an enormous effect of glyphosate in *L. rohita* embryos. The teratogenic effects of glyphosate led to deformed embryos, decreased heart rate, and high mortality in a dose-dependent manner. This chemical would also adversely affect the natural population of the carps and pond culture system which receives runoff from agricultural land. To sustain the production of food fish from aquaculture, an amelioration mechanism (feed supplementation) can be developed further for combating the impact of the herbicide.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

All ethical guidelines of ICAR-CIFA, Bhubaneswar, India, were followed for the handling of animals in this experiment. The experimental trial was conducted according to OECD 2019 guidelines.

Disclosure

Swati Sucharita Panda and Ipsita Iswari Das are co-first author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Swati Sucharita Panda wrote the manuscript. Ipsita Iswari Das and Gouranga Biswas reviewed and edited the manuscript. Swati Sucharita Panda, Ipsita Iswari Das, and Jitendra Kumar Sundaray conducted the experiment. Jitendra Kumar Sundaray was responsible for conceptualization, supervision, and funding acquisition. Rajesh Kumar supervised the work. Lakshman Sahoo reviewed the manuscript. Swati Sucharita Panda and Ipsita Iswari Das contributed equally to this study.

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