EMBRYOTOXICITY OF CHLORPYRIFOS ON GASTRULATION, SEGMENTATION, AND HATCHING OF *CLARIAS GARIEPINUS* (BURCHELL, 1822)

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ABSTRACT

This study aimed to investigate the effect of chlorpyrifos on the embryonic development of Clarias gariepinus. Freshly fertilized eggs of C. gariepinus were subjected to varying concentrations of chlorpyrifos (0.01, 0.1, 1.0, and 10 µg L^{-1}) during embryogenesis in a static renewal bioassay. The investigation focused on three crucial stages of embryogenesis: gastrulation, segmentation, and hatching. During the gastrulation stage, observations of germ rings, caudal edge, and cephalic edge were consistent across both treatment and control groups. Results from the segmentation stage revealed complete somite blocks in both treatment and control groups. At hatching, the optic primordial, myotomal muscle, yolk sac, notochord, and mouth gape were fully formed at 24 hours post-hatching in the control and treatment groups, except at the highest concentration (10 µg L^{-1}), where hatching did not occur. Notably, there were no significant aberrations during the embryogenesis period across the treatments. The hatchability test indicated that at the lowest concentration of chlorpyrifos (0.01 µg L^{-1}), 60% of the thirty fertilized eggs hatched. The 0.1 µg L^{-1} treatment exhibited a hatching rate of 40%, while the 1.0 µg L^{-1} treatment group showed a hatching rate of 20%. Although exposure of catfish embryos to chlorpyrifos at reduced concentrations did not result in significant effects, except at 10 µg L^{-1}, the mere presence of chlorpyrifos in water bodies underscores the need for further evaluation of the subtle effects at environmentally relevant concentrations, which can only be identified through mechanistic analysis, on the development of fish eggs, larvae, and other aquatic fauna.

Keywords: Chlorpyrifos, Embryogenesis, Gastrulation, Segmentation, Hatching, *Clarias gariepinus*

INTRODUCTION

In Nigeria, the need for agricultural intensification arises from the country's burgeoning population, which has led to both land scarcity and increased demand for food. In response to the escalating need for food, farmers across the nation cultivate high-yielding crop varieties. However, these varieties are highly vulnerable to various pests and diseases. Consequently, farmers resort to the use of pesticides to safeguard their crops, enhance yields, and improve the quality of their produce (Maton *et al.*, 2016). The Nigerian government, like many other developing nations, advocates for the widespread use of pesticides to boost agricultural productivity (Sarkar *et al.*, 2021). Currently, farmers employ various pesticides, with chlorpyrifos being a prominent choice (Shahjahan *et al.*, 2017). Chlorpyrifos (0,0-diethyl 0-3,5,6-trichloro-2-pyridyl phosphorothioate, CAS No. 2921-88-2) is a synthetic organophosphate insecticide and acaricide widely utilized in both agricultural and household settings (Yen *et al.*,

2011). Farmers rely on this insecticide to control pests affecting crops such as rice, coconut, and vegetables like beans and potatoes. Its mode of action involves poisoning the stomach of pests and inhibiting enzyme activity by binding to the enzyme acetylcholinesterase (AChE) through phosphorylation (Jin *et al.*, 2015). Chlorpyrifos exhibits relatively prolonged persistence in the environment compared to other organophosphorus insecticides, with a half-life in water-sediment systems ranging from 29 to 74 days (Palma *et al.*, 2009). This persistence contributes to its adverse impact on non-target aquatic organisms, encompassing both vertebrates and invertebrates, resulting in high toxicity (Jin *et al.*, 2015; Rubach *et al.*, 2012).

The African catfish, *Clarias gariepinus* Burchell 1822 (Siluriformes: Clariidae), ranks among the most widely distributed freshwater fish species in Africa, thriving in tropical swamps, lakes and rivers. In 2018, global fish production reached 179 million tonnes, with 156 million tonnes allocated for human consumption, approximately translating to an estimated annual supply of 20.5 kg of fish per capita (FAO, 2020). Developing countries host around 94% of all freshwater fish species, contributing over 6% to the world's animal protein supply for human consumption (FAO, 2007). However, a significant portion of these fish and other aquatic organisms consumed by humans reside in rivers, which serve as the ultimate repositories for pollutants originating from spray drift, runoff, and leaching. Chlorpyrifos-contaminated soils exacerbate the situation, causing hazardous impacts on the aquatic environment (Agbohessi *et al.*, 2013). Pesticide pollution poses toxicological threats to non-target aquatic organisms in the environment.

Assessing alterations in organisms at the developmental, physiological, biochemical, or molecular levels provides biomarker tools crucial for monitoring environmental quality. The embryonic or larval stage of developing fish is generally considered the most sensitive in the life cycle of teleost fish, particularly vulnerable to low levels of environmental pollutants. Biomarkers serve as measurable indicators of the interaction between environmental xenobiotics and biological effects. Inhibiting or inducing these biomarkers proves to be an effective approach to measuring potential impacts of pollutants on aquatic organisms. In Nigeria, most catfish farms are situated in and around agricultural fields, with their water sources frequently coming into contact with the paddy ecosystem where pesticides and insecticides are routinely employed. The African catfish, known for its high fecundity, is a species that can be easily bred and maintained in laboratory settings (Marimuthu *et al.*, 2013).

Zebrafish exposed to chlorpyrifos during early development exhibit enduring neurobehavioral deficits, with adult behavioural impairments resulting from their early exposure to the pesticide (Levin *et al.*, 2004). Their earlier research discovered that embryonic exposure of zebrafish to chlorpyrifos results in significant impairments in discrimination learning and swimming speed (Levin *et al.*, 2003).

Furthermore, exposure of zebrafish embryos to 2,4-dichlorophenoxyacetic acid led to heightened mortality, reduced hatching rates, and pericardial sac extension (Li *et al.*, 2017). Other studies on zebrafish embryos exposed to 2,4-dichlorophenoxyacetic acid reveal extended pericardial sacs, tail deformities, altered swim behaviour, increased mortality, and neural defects causing reduced vision (Dehnert *et al.*, 2019). Despite the short developmental time, ease of cultivation, and year-round reproduction of the African catfish, there is insufficient information about its fertilization mechanisms, embryonic development, and ontogenetic progression. In addition to being an excellent candidate for aquaculture, *C. gariepinus* has played a role in fundamental research and toxicological studies. These studies underscore the importance of utilizing various developmental stages in toxicity testing, emphasizing that relying solely on one stage is insufficient for accurate chemical risk assessments. Therefore, this study aimed to elucidate the embryonic development of *C. gariepinus*, enhancing our understanding of its early life history. The findings from these studies will contribute to discussions and interpretations of toxicological investigations. This investigation specifically focuses on evaluating the impact of chlorpyrifos

on the developmental stages of *C. gariepinus*, spanning from gastrulation to hatching.

MATERIALS AND METHODS

Collection and Cultural Condition: *C. gariepinus* male and female brood stocks of two-month-old each with initial weights of 2 and 3 kg respectively were obtained from the Department of Fisheries, Faculty of Agriculture, University of Benin in Benin City, Edo State, Nigeria. This experiment was carried out at the Animal House Laboratory, Department of Animal and Environmental Biology, Faculty of Life Sciences, University of Benin. The fish used for spawning were acclimatized and maintained in a 60-litre tank capacity for two days before the experiment. The matured male possessed prominent, pinkish-colored pointed genital papillae (Olaniyi and Omitogun, 2013) and the females were gravid with a swollen abdomen that freely oozed out eggs upon gentle pressure on their abdomen from their pinkish or reddish swollen vent (Olaniyi and Omitogun, 2013).

Test Substances

Chlorpyrifos: Chlorpyrifos is a broad-spectrum, organophosphate (OP) insecticide, acaricide, and nematicide. Chlorpyrifos is the name for the chemical, O, O-diethyl O-3,5,6-trichloro-2-pyridinyl phosphorothioate. The molecular weight is 350.6 g/mol and solubility is 0.0014 g/L (1.4 mg/L) at 25^{o}C.

Test water: The water used for the bioassay was dechlorinated tap water. The dechlorination of the test water was done by allowing it to stand exposed for 36 hours (Ezemonye and Enuneku, 2005). The dechlorinated water was used for the acclimatization of the catfish, in the control and the treatment tanks.

Water quality: The water quality was monitored during the acclimatization, acute and sub-lethal bioassay procedures. Physicochemical parameters such as temperature, pH, dissolved oxygen (DO), turbidity, total alkalinity, and conductivity were measured using standard methods (Baird *et al.*, 2017).

Experimental Procedure

Spawning: The female fish was injected with ovaprim (gonadotropic hormone) intramuscularly at 0.5 ml/kg catfish body weight between the dorsal fin and the lateral line, just below the anterior part of the dorsal fins using a graduated syringe (2.5 ml). After injection, slight pressure was applied with the finger around the point of insertion to allow for proper distribution of the hormone suspension throughout the muscles. After 10 hours of the latency period, the female fish was stripped by applying slight pressure on the abdomen. Subsequently, the male catfish was euthanized to extract its gonads, from which milt was then expressed. The wet method of fertilization was used, where the eggs were mixed with diluted sperm in a saline solution (0.9% NaCl), as described by Olaniyi (2013). Following exposure to different chlorpyrifos concentrations, samples were collected every 10 minutes for the initial three hours, then hourly for the subsequent nine hours, and finally at two-hour intervals for the ensuing 12 hours until approximately 26 hours post-exposure when hatching occurred.

Toxicity test: The fertilized eggs were exposed to four different concentrations of chlorpyrifos and labelled appropriately as CHL_0 (0.00 µg/L), CHL_1 (0.01 µg/L), CHL_2 (0.1 µg/L), CHL_3 (1.0 µg/L), and CHL_4 (10 µg/L). Thirty eggs each were counted and removed from the original mixture and spread on a netting substrate in smaller bowls containing 2 litres of water to use for further studies such as fertilization rate, hatchability, and survival rate. This was done for each of the egg-sperm mixtures of the five concentrations of chlorpyrifos, CHL_0 (0.00 µg/L) was the control. The experiment was conducted in triplicates.

Estimation of Percentage Hatchability: Following fertilization, the eggs were then transferred to their original lot for hatching. After hatching, the numbers of hatchlings within each batch were carefully counted and the hatching rate was calculated using the following equation:

Hatching rate = Number of eggs hatched / Total number of eggs in a batch x 100 (Adebayo, 2006).

Embryonic Studies and Photomicrography: The 30 fertilized eggs were removed randomly from the incubating tanks into a Petri dish in 3 batches of 10 eggs per batch. Embryonic development was monitored, observed, photographed, and documented live with the aid of a light microscope (Unico Binocular Microscope G380) fitted with a digital camera connected to a laptop computer. Selected eggs were viewed under the microscope immediately after fertilization at 5-minute intervals for the first 3 hours, later at an hourly and two hourly intervals until hatching. The developmental stages of eggs starting from first cleavage to hatching were examined microscopically at a magnification of x1000 for 22 hours. Photomicrographs were used to describe important stages of gastrulation, somite formation of embryo, and hatching. The accurate timing and detailed description of each stage were recorded.

RESULTS AND DISCUSSION

Physicochemical Properties of the Test Media: The results of the physicochemical parameters were almost the same across treatment groups (Table 1) and were within the desirable range for fish culture throughout the duration of the experiment. The parameters showed no significant differences ($p>0.05$) from the control. The observed stability of the water quality characteristics showed that chlorpyrifos did not adversely reduce the water quality in the treatment tanks. Our findings were consistent with the report of Papoulias *et al.* (2014) on the exposure of Japanese medaka (*Oryzias latipes*) to atrazine-polluted water. They reported that atrazine exposure to *O. latipes* measured with ELISA remained fairly constant and near nominal concentrations for the entire duration of the experiment.

Morphological Events During Embryonic Development

Fertilization to hatching: At 27^0C it was observed that fertilized eggs were sticky, transparent, and spherical with a mean diameter of 1.10 ± 0.21 mm. The timing of gastrula, segmentation, and hatching in the 27^0C control tanks was similar to those reported earlier by Agbohessi *et al.* (2022) at 27^0C. The similarities in the timing of developmental events may be due to temperature similarities. The summary of the various stages and timing of *C. gariepinus* exposure to different concentrations of chlorpyrifos during embryonic development at 27^0C has been presented in Table 2. Gastrulation in the control group occurred 7 hours after fertilization with the embryo having germ rings, cephalic and caudal edges which were observed at the advanced stages of the blastula (Figure 1). At the segmentation stage, somite was formed at 12 hours post-fertilization (Figure 2). The development and maturation of somite blocks and the body pigmentation started at the cephalic parts of the embryo and progressed caudally. Hatching occurred at 24 hours post-fertilization (Figure 3) which differed slightly from those reported by Opute and Oboh (2020) at 22 hours post-fertilization. This observed difference may be attributed to the differences in chemical mode of action between atrazine and chlorpyrifos and/or concentrations of exposure (Wang *et al.,* 2020). At the time of hatching a violent movement of the tail to either side against the chorion walls was observed followed by contraction as a result of which the chorion walls were broken.

Post-Fertilization Stage of Embryonic Development: The developing fish embryo and early larval stages are especially sensitive indicators of many types of pollution in the aquatic ecosystem (Hallare *et al.,* 2005). The sensitivity of embryonic stages to chemical-induced adverse effects is based on the occurrence of developmental events such as cleavage, morulation, blastulation, gastrulation, and organogenesis (Navis *et al.,* 2013). Sule and Adikwu (2004) reported gastrulation at 7 hours 10 minutes and hatching at 22 hours in *C. gariepinus* under laboratory conditions. In this study, just like in the control group, all the treatment groups exposed to chlorpyrifos at 7 hours post-fertilization showed germ rings, cephalic edge, and caudal edge formation during gastrulation.

Table 1: Concentrations of the physicochemical parameters of the test media of *Clarias gariepinus* during embryonic development

Parameter	Concentration				
	0.00 µg/L	0.01 µg/L	0.1 µg/L	1.0 µg/L	10 µg/L
Temperature (^{0}C)	27.37 ± 0.39	27.39 ± 0.39	27.41 ± 0.37	27.38 ± 0.35	27.46 ± 0.35
pH	7.02 ± 0.09	7.00 ± 0.08	7.00 ± 0.11	7.03 ± 0.12	7.04 ± 0.13
Conductivity (µS/cm)	102.20 ± 0.92	102.30 ± 0.95	102.00 ± 0.95	102.20 ± 0.92	101.10 ± 0.88
Turbidity (mg/l)	0.23 ± 0.02	0.24 ± 0.03	0.24 ± 0.03	0.24 ± 0.02	0.23 ± 0.02
Alkalinity (mg/l)	17.43 ± 0.43	17.55 ± 0.36	17.53 ± 0.43	17.46 ± 0.49	17.50 ± 0.44
Hardness((mg/l))	32.52 ± 0.37	32.57 ± 0.40	32.48 ± 0.33	32.46 ± 0.33	32.50 ± 0.34
DO (mg/l)	8.11 ± 0.12	8.13 ± 0.13	8.12 ± 0.13	8.14 ± 0.10	8.12 ± 0.14

Treatment means for all the parameters were not significantly different (p>0.05)

Table 2: Summary of Embryonic development from Gastrulation to Hatching of *Clarias gariepinus*

S/N	Stages of development	Time after fertilization	Description
1.	Gastrulation stage	7 hours	The embryo develops germ rings and cephalic and caudal edges observed at the blastula's advanced stages.
2.	Somite formation	12 hours	At 12 hours post-fertilization, the development and maturation of somite blocks and the body pigmentation started at the cephalic parts of the embryo and progressed caudally.
3	Hatching	24 hours	The violent movement of the tail to either side against the chorion walls is followed by contraction as a result of which the chorion wall breaks and hatching occurs.

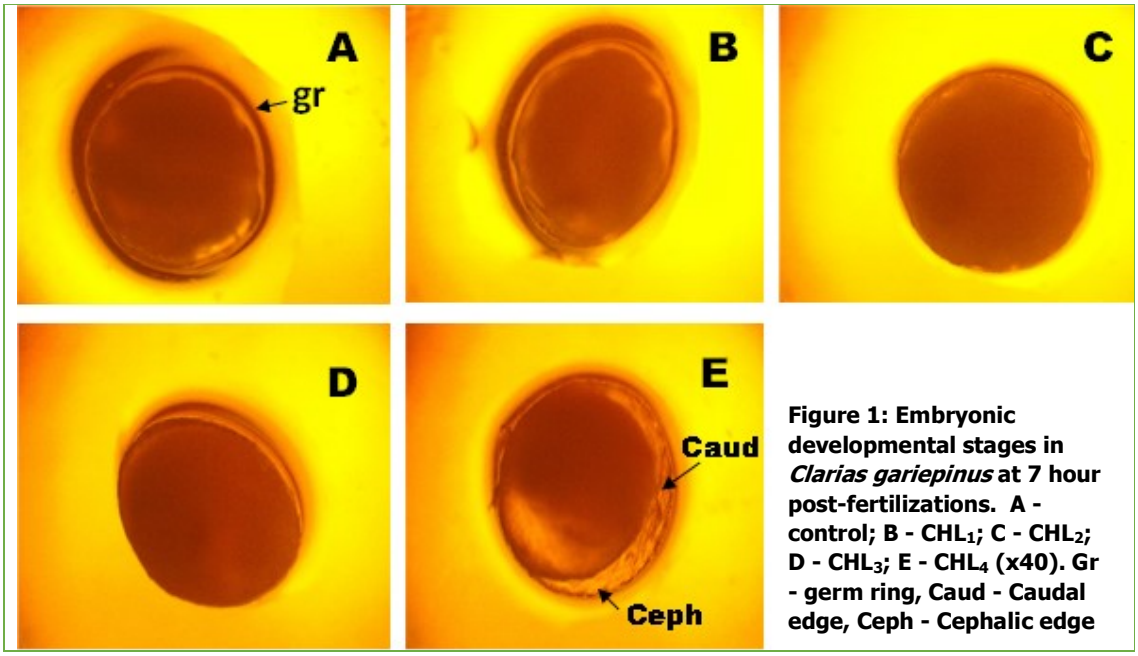

Figure 1: Embryonic developmental stages in *Clarias gariepinus* at 7 hour post-fertilizations. A - control; B - CHL_1; C - CHL_2; D - CHL_3; E - CHL_4 (x40). Gr - germ ring, Caud - Caudal edge, Ceph - Cephalic edge

Similarly, results from the segmentation stage showed complete somite blocks across the treatment groups and control. At lower concentrations, atrazine has been reported to delay hatching without completely inhibiting the processes of gastrulation and segmentation when exposed to *C. gariepinus* at 7 hours post-fertilization (Opute and Oboh, 2020). At hatching, it was observed that optic primordial, myotomal muscle, yolk sac, notochord, and

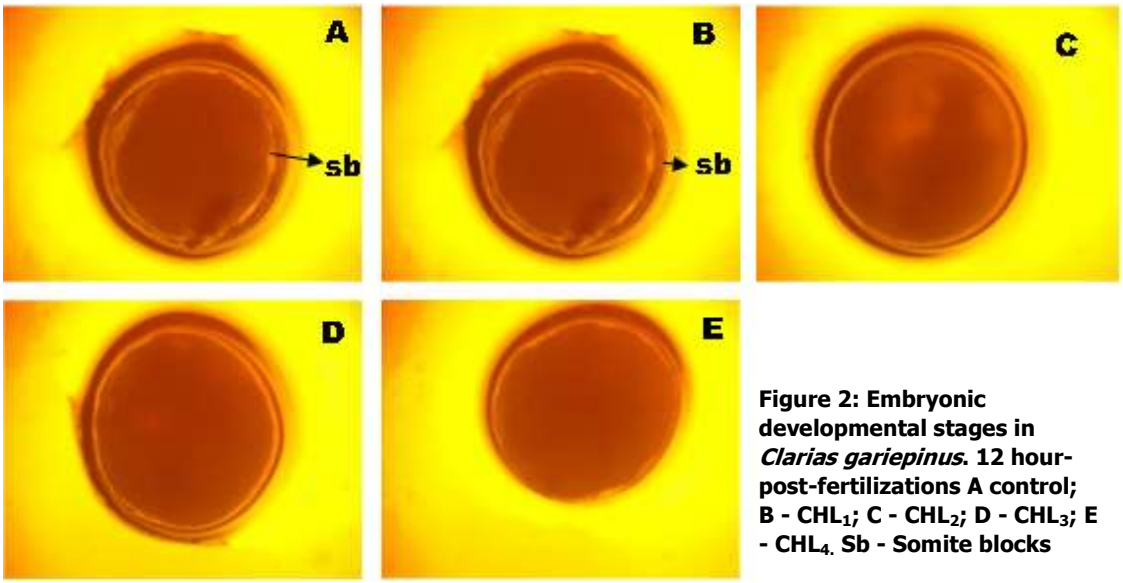

Figure 2: Embryonic developmental stages in *Clarias gariepinus*. 12 hour-post-fertilizations A control; B - CHL_1; C - CHL_2; D - CHL_3; E - CHL_4. Sb - Somite blocks

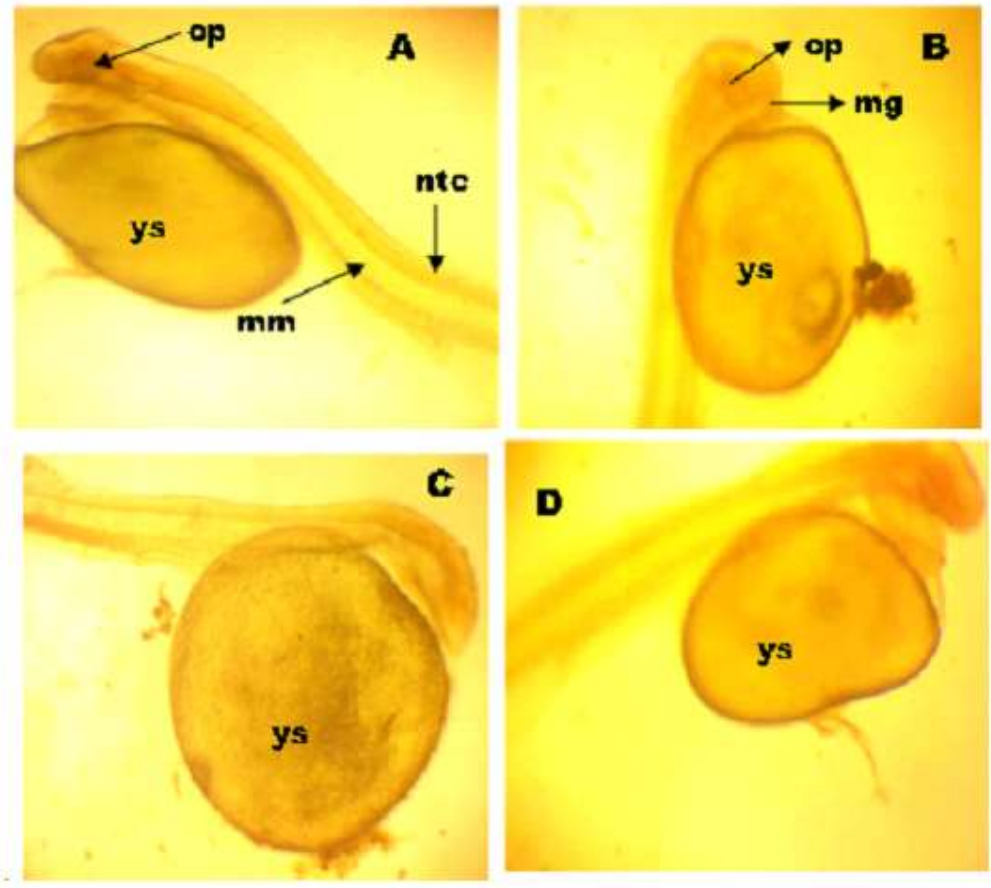

Figure 3: Embryonic developmental stages in *Clarias gariepinus* 24 hour-post-fertilization. A - control; B - CHL_1; C - CHL_2; D - CHL_3. Op - optic primordium, mm - myotomal muscle, ys - yolk sac, ntc - notochord, mg - mouth gape

mouth gape was fully formed at 24 hours post-hatching in the control and for all treatments except at the highest concentration (10 µg/L) where hatching was not observed. Thus, there was no significant aberration ($p>0.05$) during the period of embryogenesis across the treatments.

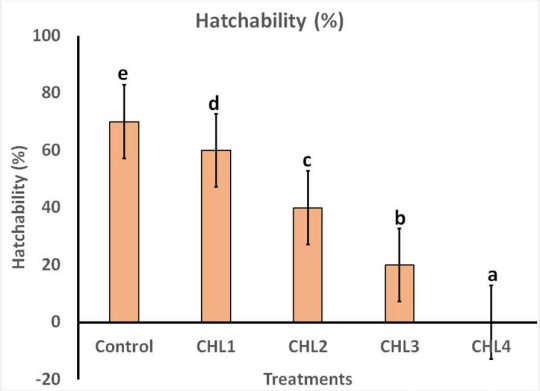

Figure 4: Graphical representation of percentage hatchability of *Clarias gariepinus* embryo exposed to chlorpyrifos

This study was in agreement with earlier findings on *Trichogaster fasciata* (banded gourami) exposed to 0.01 to 100 µg/L concentrations of chlorpyrifos. It was reported that there was no malformation at the embryonic stage (Sumon *et al.,* 2019).

Larval Development (Hatching): This study revealed that exposure to varying concentrations of chlorpyrifos adversely impacts the hatching success, incubation duration, and mortality rates of *C. gariepinus* embryos and larvae. There was a significant decline in hatching success as the chlorpyrifos concentration increased. Specifically, in the control tank, hatching commenced 24 hours post-fertilization with a 70% embryo hatch rate. Conversely, in the treatment groups, the hatch rates were 60, 40, and 20%, in the 0.01, 0.1, and 1.0 µg/L groups respectively. No hatching was observed at 10.0 µg/L. Several studies have indicated that the fish chorion offers little protection to embryos exposed to various pesticides (Ansari and Ahmad, 2010). The chorion of fertilized eggs, when submerged in water, allows the passage of lipophilic molecules with elevated n-octanol-water partition coefficients (Log Kow). Pollutants with higher Log Kow values can more easily permeate the chorion than those with lower values (Agbohessi *et al.*, 2013).

In this study, chlorpyrifos demonstrated its lipophilic properties. It was evident that the molecules of the test chemical swiftly permeated the chorion, a structure with a phospholipid composition. As the concentration of these molecules in the solution increased, their penetration through the chorion also increased. Consequently, the toxicity level of chlorpyrifos within the eggs was directly related to its concentration (Tyor and Harkrishan, 2016; Agbohessi *et al.*, 2020). This phenomenon is exemplified by the observed 100% mortality rate of eggs/embryos in the highest treatment concentration (10.0 µg/L). Furthermore, the mortality rates of these embryos increased proportionally with increasing concentrations.

This pattern has been documented by Prastika *et al.* (2021) in silver rasbora (*Rasbora argyrotaenia*) eggs exposed to organophosphates. Rahman *et al.* (2020) with Zebrafish (*Danio rerio*) eggs exposed to sumithion, and Tyor and Harkrishan (2016) with common carp (*Cyprinus carpio*) eggs exposed to imidacloprid. Similar results have also been reported by Agbohessi *et al.* (2013) with *C. gariepinus* eggs exposed to acute concentrations of Endosulfan and Tihan 175 O-TEQ.

The hatching rates observed in the chlorpyrifos treatments (CH_1 to CH_3) in this study ranged between 20 to 60% (Figure 4). These rates were notably lower than the 62.2 to 75.9% observed in *C. gariepinus* eggs/embryos exposed to Pyro FTE 472 (Agbohessi *et al.*, 2022) but higher than the 0.12 to 68.9% observed in those exposed to buprofezin (Marimuthu *et al.*, 2013) and the 3.3% in eggs/embryos exposed to Atrazine (Opute and Oboh, 2020).

In the natural hatching process of fish embryos, the chorion undergoes digestion due to the secretion of a proteolytic enzyme from the embryo's hatching gland cells (Marimuthu *et al.*, 2013). Pollutant exposure can potentially alter this hatching mechanism by influencing the secretion of the proteolytic enzyme. Additionally, the embryo's movements within the chorion can facilitate hatching (Agbohessi *et al.*, 2013). In summary, there was a significant decrease in hatching rates with increasing concentrations of chlorpyrifos. This observation is similar to the findings from several studies (Tyor and Harkrishan, 2016; De la Paz *et al.*, 2017; Rahman *et al.*, 2020 and Opute and Oboh, 2020), all of whom reported decreased hatching success in various fish species due to pollutant exposures.

Conclusion: The results from this study have demonstrated clearly that exposure of *C. gariepinus* eggs to chlorpyrifos showed no significant disruptive aberration in the gastrulation and segmentation stages of embryonic development. The hatching rate was however observed to reduce with increasing concentrations of chlorpyrifos. Chlorpyrifos, therefore, has serious reproductive implications which may lead to a declining fish population in the wild if the unsafe application of chlorpyrifos is left unchecked. Furthermore, studies should be carried out at the molecular level to better understand the subtle effects of these insecticides on the embryonic development of *C. gariepinus* especially in terms of neurotransmission and neurodevelopment.

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