Chronic Sub-Lethal Glyphosate Exposure Induces Endocrine Disruption, Oxidative Stress, and Multi-Generational Reproductive Impairment in the African Catfish (*Clarias Gariepinus*)

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ABSTRACT

Glyphosate-based herbicides are major contaminants in aquatic environments, yet the reproductive and Tran's generational consequences of chronic sub-lethal exposure to them remain poorly researched in the study area. We therefore, evaluated the physiological, endocrine and multigenerational reproductive effects of chronic sub-lethal exposure to glyphosate in African catfish (Clarias gariepinus). Sexually mature fish (n = 120) were exposed to environmentally relevant concentrations of glyphosate (0, 5, and 25 mg/L) for 60 days. Growth, behaviour, haematological indices, plasma cortisol and glucose, reproductive hormones (17β-estradiol, testosterone, and 11-ketotestosterone), hepatic oxidative stress markers (malondialdehyde, superoxide dismutase, catalase, and glutathione), gonadosomatic and hepatosomatic indices, gamete quality, and in vitro fertilization success were assessed. Tran's generational effects were evaluated by monitoring survival, deformities, and growth of F1 larvae for 7 days post-hatch. Data were analysed using ANOVA, principal component analysis, and dose-response regression. Glyphosate exposure induced a pronounced dose-dependent stress response, with plasma cortisol increasing from 15.8 ± 1.2 to 78.9 ± 3.8 ng/mL ($p < 0.001$), accompanied by endocrine disruption characterized by male feminization and female masculinization. Hepatic oxidative stress was severe, evidenced by a 4.3-fold increase in malondialdehyde and compensatory antioxidant enzyme activation. Gonadal integrity was markedly impaired, with gonadosomatic index reductions exceeding 50% in both sexes, spermatogenic arrest, and increased oocyte atresia. Fertilization and hatchability declined by 64%

and 70%, respectively, while unexposed F1 offspring exhibited reduced survival, increased deformities, and impaired growth. It is therefore concluded that chronic sub-lethal glyphosate exposure compromises reproductive capacity and induces Tran's generational fitness deficits with potential population-level consequences.

KEYWORDS
Glyphosate, Clarias gariepinus, reproductive toxicity, endocrine disruption, oxidative stress, Tran's generational effects

I. INTRODUCTION

Glyphosate (N-(phosphonomethyl) glycine) is the active ingredient in the world's most widely used broad-spectrum herbicide formulations, with global application exceeding 800,000 metric tons annually (Benbrook, 2016). Its extensive use in agriculture, aquaculture, and urban settings has led to ubiquitous environmental distribution, with surface water concentrations frequently detected in the µg/L to mg/L range following runoff events. Despite its purported rapid degradation and low soil mobility, glyphosate and its primary metabolite, aminomethylphosphonic acid (AMPA), persist in aquatic ecosystems, posing potential risks to non-target aquatic organisms (Van Bruggen *et al.,*2018).

Traditional Eco toxicological assessments have predominantly focused on acute lethality (LC_{50} values), with reported 96-hour LC_{50} values for fish ranging from 10 to 140 mg/L depending on species and formulation. However, growing evidence suggests that sub-lethal concentrations, often orders of magnitude below acutely toxic levels, can induce significant physiological disturbances through chronic exposure pathways. These sub-lethal effects may have more profound ecological consequences than acute toxicity by impairing critical life functions such as reproduction, growth, and behaviour, ultimately affecting population dynamics and community structure.

Clarias gariepinus, the African sharp tooth catfish, represents an ecologically and economically significant freshwater species distributed throughout Africa and parts of Asia. Its physiological resilience, rapid growth, and year-round reproductive capacity in captivity have established it as a valuable species for aquaculture. Eco toxicologically, *C. gariepinus* serves as an excellent sentinel species due to its benthic feeding behaviour, high lipid content (facilitating bioaccumulation), and sensitivity to environmental contaminants. Furthermore, its well-characterized reproductive biology, including external fertilization and synchronous oocyte development, enables detailed assessment of reproductive endpoints.

Although numerous studies on glyphosate toxicity exist, significant knowledge gaps remain regarding:

[1] The integrated pathway from molecular stress to reproductive impairment.
[2] Sex-specific vulnerabilities to chronic exposure.
[3] The potential for Tran's generational effects.
[4] The concentration-response relationships for sub-lethal reproductive endpoints. Most research works employ single-endpoint approaches which lack the comprehensive integration necessary for ecological risk assessment.

This study examined chronic sub-lethal glyphosate effects on reproduction in *Clarias gariepinus* across three integrated compartments:

[1] Endocrine and oxidative mechanisms.
[2] Gonadal and gamete integrity.
[3] Reproductive performance and F_1 offspring fitness, establishing dose–response relationships and an adverse outcome pathway for reproductive toxicity. It also provides data for ecological risk assessment and contributes to the understanding of the mechanisms underlying herbicide-induced reproductive failure in aquatic vertebrates.

II. MATERIALS AND METHODS

A. Experimental Animals and Acclimatization

A total of 120 sexually mature *Clarias gariepinus* (mean weight: 1.2 ± 0.2 kg; mean total length: 45.3 ± 3.1 cm; age: 12-15 months) were procured from a certified commercial hatchery (SegKat Farm Ltd, Ibadan, Oyo State, Nigeria). Fish were transported in oxygenated polyethylene bags and gradually acclimatized to laboratory conditions over 72 hours. Initial health screening included visual inspection for parasites, lesions, or abnormal behaviour; only clinically healthy specimens were selected.

Fish were maintained in a 2000-L fiberglass holding tank with a recirculating aquaculture system (RAS) for 21 days prior to experimentation. Water parameters were maintained at: temperature 26.0 ± 0.5°C (optimal for *C. gariepinus* reproduction), pH 7.2 ± 0.2, dissolved oxygen >6.0 mg/L, photoperiod 12L: 12D, ammonia <0.02 mg/L, nitrite <0.1 mg/L, and nitrate <20 mg/L using biological filtration and weekly 30% water exchanges. Fish were fed a commercial pelleted diet (Raanan Fish Feed, Israel; 35% crude protein, 8% lipid) at 3% body weight daily, with feeding suspended 24 hours prior to any handling or sampling.

B. Experimental Design and Treatment Groups

A completely randomized design with three treatments in triplicate was employed:

[1] Control Group (C): 0 mg/L glyphosate
[2] Treatment 1 (T1): 5 mg/L glyphosate (10% of reported 96-h LC_{50})
[3] Treatment 2 (T2): 25 mg/L glyphosate (50% of reported 96-h LC_{50}

Technical-grade glyphosate (97.5% purity, Sigma-Aldrich, Germany) was used to Eliminate confounding effects from formulation surfactants. Stock solutions were prepared Weekly.

Experimental Units: Nine 500-L polyethylene tanks (effective water volume: 400 L) served as experimental units. Each tank was randomly assigned to a treatment and stocked with 10 fish (5 males + 5 females), determined by examination of the genital papilla (conical in males, rounded and reddish in females) and confirmation via cannulation where

necessary. Tank replication followed a blocked design to account for positional effects in the laboratory.

C. Exposure Regime and Husbandry

The 60-day exposure period commenced after gradual temperature and water chemistry adjustment. Glyphosate concentrations were maintained through semi-static renewal: 80% water exchange every 48 hours with re-dosing to nominal concentrations. Water quality parameters were monitored daily using a multipara meter probe (YSI Pro DSS, USA). Fish were observed twice daily for mortality, behavioural abnormalities, and feeding response. Moribund fish meeting predefined humane endpoints (cessation of feeding for >3 days, loss of equilibrium, severe lesions) were euthanized and excluded from subsequent analyses.

D. Sampling Protocol and Timeline

Growth and Behavioural Monitoring: Days 0, 30, 60: Individual fish were anesthetized (MS-222, 100 mg/L), weighed and measured.
Specific Growth Rate: $SGR = [(\ln W_2 - \ln W_1) / (t_2 - t_1)] \times 100$.
Feed Conversion Ratio: FCR = Total feed given / Total weight gain.
Condition Factor: $K = (\text{Weight} / \text{Length}^3) \times 100$.

[1] *Behavioural Analysis:*

Weekly 15-minute video recordings of each tank were analysed using EthoVision XT (Noldus, Netherlands) for: swimming velocity, percentage of time spent in erratic movement, social proximity index, and surface breathing frequency. Blood Sampling and Haematological Analysis (Days 30 & 60): Fish were anesthetized as above, and 2 or 3 mLs of blood were collected from the caudal vasculature using heparinized syringes. The blood samples were used for Haematocrit and haemoglobin determination, total and differential leukocyte counts. Plasma separated by centrifugation at 3000 × g for 15 min at 4°C; plasma stored at -80°C for biochemical and hormonal assays.

[2] *Biochemical and Hormonal Assays*

Plasma cortisol and glucose were quantified using commercial ELISA kits (Cayman Chemical, USA; intra-assay CV <8%), 17β-Estradiol, testosterone, and 11-ketotestosterone were measured using fish-specific ELISA kits (Cusabio, China; detection limits: 1.5 pg. /mL, 6.0 pg. /mL, and 3.0 pg. /mL respectively; cross-reactivity < 0.1%) while total plasma protein was determined via Bradford assay (Bio-Rad, USA).

E. Terminal Sampling and Organ Collection (Day 60)

Fish were euthanized by MS-222 overdose (300 mg/L) followed by spinal severance. Gonads and liver were excised, blotted, and weighed and the following determined.
Gonadosomatic Index: GSI = (Gonad weight / Body weight) × 100.
Hepatosomatic Index: HSI = (Liver weight / Body weight) × 100.

F. Oxidative Stress Analysis

Liver tissue (100 mg) was homogenized in cold phosphate buffer (pH 7.4) and centrifuged at 12,000 × g for 20 min at 4°C. Supernatants were analysed for Lipid Peroxidation, Antioxidant Enzymes, Non-enzymatic Antioxidant and Protein Normalization. Malondialdehyde (MDA) content via thiobarbituric acid reactive substances (TBARS) assay (Ohkawa *et al.,* 1979). Superoxide dismutase (SOD) activity via inhibition of nitro blue tetrazolium reduction (Beauchamp & Fridovich, 1971); catalase (CAT) activity via H_2O_2 decomposition (Aebi, 1984). Reduced glutathione (GSH) via Ellman's reagent (Sedlak & Lindsay, 1968). All values expressed per mg protein (Bradford method).

G. Gamete Collection and Quality Assessment

On day 60, remaining fish were induced for final maturation using synthetic GnRH analogue (Ova prim, Syndel, Canada; 0.5 mL/kg). After 12 hours. Milt was collected by gentle abdominal pressure, assessed for motility, Viability and Concentration. Computer-assisted sperm analysis (CASA, Hamilton Thorne, USA): total motility (%), progressive motility (%), curvilinear velocity (VCL). Eosin-nigrosin staining (>200 sperm counted). Haemocytometer count.

H. Egg Collection

Stripped eggs were assessed for diameter, ocular micrometre (30 eggs/female) and morphology: Normal vs. irregular shape.

I. Fertilization Assay

In vitro fertilization using pooled gametes from each tank (eggs from 5 females + milt from 5 males). Fertilization rate assessed at 4 hours post-fertilization (hpf) by cleavage observation.

J. Embryonic Development and Larval Fitness (F1 Generation)

Fertilized eggs from each tank replicate were incubated in separate 10-L aquaria with glyphosate-free water (26°C). Assessments included Hatchability and Larval Rearing. Percentage of hatched larvae at 24-36 hpf. Larvae were reared for 7 days post-hatch (dph) with *Artemia* nauplii. Fitness was assessed through Survival at 7 dph, Total length and yolk sac diameter (Image software), Deformity assessment: skeletal (lordships, scoliosis), craniofacial, enema and Swimming performance: escape response to tactile stimulus.

K. Statistical Analysis

All statistical analyses were performed using R v4.1.2 (R Core Team, 2021) and Graph Pad Prism v9.0. Normality (Shapiro-Walk test) and homogeneity of variance (Levine's test) were verified. Data were analysed using Two-way ANOVA: Treatment × Sex interaction for growth, hormones, and organ indices. Repeated Measures ANOVA: Time-series data (growth, behaviour). One-way ANOVA with Turkey's HSD: Treatment effects within sexes. Principal Component Analysis (PCA): Dimension reduction of multivariate data. Correlation Analysis: Pearson's or Spearman's correlation matrices. Regression Analysis: Dose-response modelling (linear, exponential, logistic). Effective Concentration Calculation: EC_{10}, EC_{25}, EC_{50} via four-parameter logistic regression. Survival Analysis: Kaplan-Meier with log-rank test for larval survival. Significance was set at $\alpha = 0.05$. Data presented as mean ± standard error of mean (SEM). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC Protocol #AF-2023-017) and followed OECD Guideline 229 for fish reproductive testing.

III. RESULTS

A. Survival and Clinical Observations

Survival was 100% in the control group throughout the 60-day exposure. The T1 (5 mg/L) group exhibited 93.3% survival (2 mortalities on days 45 and 52), while T2 (25 mg/L) showed significantly reduced survival of 80% (6 mortalities between days 30-60; $p<0.01$, Kaplan-Meier log-rank test). Mortalities were preceded by clinical signs including lethargy, anorexia, loss of equilibrium, and darkening of skin pigmentation.

B. Growth Metrics

Glyphosate exposure induced a dose-dependent suppression of growth (Table 1). Final body weights in T2 males and females were reduced by 10.4% and 12.0% respectively compared to controls ($p<0.001$). Specific Growth Rate (SGR) showed significant treatment ($F_{2, 90} = 85.42$, $p<0.001$) and sex ($F_{1, 90} = 8.76$, $p<0.01$) effects, with a significant interaction ($F_{2, 90} = 5.23$, $p<0.01$), indicating sexually dimorphic growth inhibition. Feed Conversion Ratio (FCR) deteriorated progressively with increasing concentration, indicating reduced feed utilization efficiency.

Table 1: Growth Performance of *C*. gariepinus exposed to glyphosate for 60 days

Parameter	Sex	Control	T1 (5 mg/L)	T2 (25 mg/L)	p-value (Treatment)
Initial Wt (g)	♂	1185 ± 28	1200 ± 25	1190 ± 32	0.892
	♀	1253 ± 24	1220 ± 26	1235 ± 29	0.634
Final Wt (g)	♂	1353 ± 31	1302 ± 34*	1212 ± 38**	<0.001
	♀	1424 ± 29	1320 ± 32*	1250 ± 36**	<0.001
SGR (%/day)	♂	0.21 ± 0.01	0.14 ± 0.01*	0.03 ± 0.01**	<0.001

	♀	0.20 ± 0.01	0.13 ± 0.01*	0.02 ± 0.01**	<0.001
FCR	♂	1.82 ± 0.04	2.12 ± 0.06*	2.85 ± 0.10**	<0.001
	♀	1.88 ± 0.05	2.23 ± 0.07*	3.00 ± 0.12**	<0.001
Condition Factor	♂	1.45 ± 0.03	1.32 ± 0.04*	1.18 ± 0.05**	<0.001
	♀	1.52 ± 0.03	1.38 ± 0.04*	1.22 ± 0.05**	<0.001

Significantly different from control ($p<0.05$, Tukey's HSD)
Significantly different from control and T1 ($p<0.01$)

C. Behavioural Alterations

Quantitative behavioural analysis revealed significant treatment effects (Figure 1). Swimming velocity decreased by 35% in T1 and 62% in T2 ($p<0.001$). Erratic swimming behaviour (rapid, uncoordinated movements) increased from 4.2% of observation time in controls to 28.5% in T2 ($p<0.001$). Social proximity index (indicative of schooling behaviour) decreased dose-dependently, with T2 fish showing predominantly solitary behaviour. Surface breathing frequency increased 3.8-fold in T2, suggesting respiratory distress or hypoxia tolerance impairment.

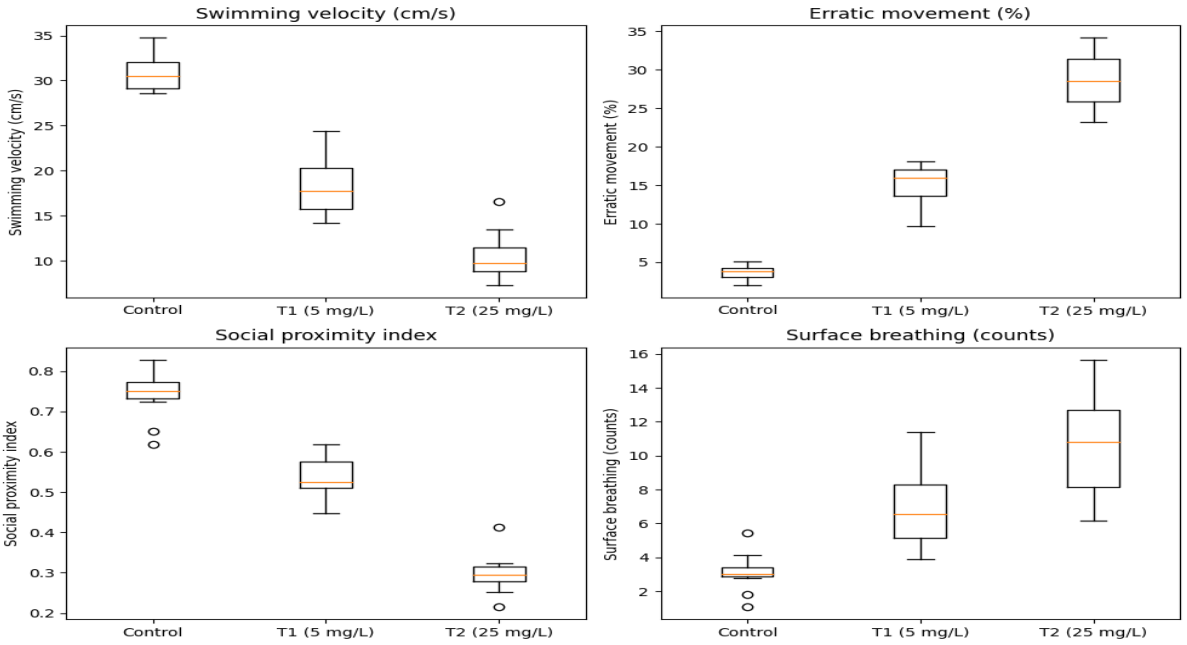

Figure 1: Quantitative analysis of behavioural parameters including (a) swimming velocity, (b) percentage of erratic movement, (c) social proximity index, and (d) surface breathing frequency across treatment groups of *C. gariepinus* exposed to glyphosate for 60 days.

D. Haematological Parameters

Haematological indices showed significant alterations consistent with stress and possible anaemia (Table 2). Haematocrit and haemoglobin concentrations decreased dose-dependently, with T2 values 25.2% and 29.6% lower than controls respectively ($p<0.001$). Total leukocyte count increased, primarily due to lymphocytosis and neutrophil, suggesting inflammatory response or immunomodulation.

Table 2: Haematological Parameters of *C. gariepinus* exposed to glyphosate for 60 days at day 60

Parameter	Control	T1 (5 mg/L)	T2 (25 mg/L)	p-value
Haematocrit (%)	32.5 ± 0.8	28.6 ± 0.9*	24.3 ± 1.2**	<0.001
Haemoglobin (g/dL)	9.8 ± 0.3	8.3 ± 0.4*	6.9 ± 0.5**	<0.001
RBC (10^6/µL)	2.85 ± 0.12	2.42 ± 0.15*	2.05 ± 0.18**	<0.001
WBC (10^3/µL)	85.2 ± 3.5	114.5 ± 4.8*	145.2 ± 6.2**	<0.001
Lymphocytes (%)	65.2 ± 3.2	58.5 ± 4.1	45.8 ± 5.2**	<0.01
Neutrophils (%)	22.5 ± 2.8	30.2 ± 3.5*	42.8 ± 4.8**	<0.001

E. Plasma Stress Markers

Plasma cortisol exhibited a strong dose-response relationship, increasing from 15.8 ± 1.2 ng/mL in controls to 78.9 ± 3.8 ng/mL in T2 (5-fold increase; $p<0.001$). Glucose levels showed parallel elevation (65.2 ± 2.1 to 122.0 ± 4.5 mg/dL; $p<0.001$), indicating chronic stress-mediated hyperglycaemia. Total plasma protein decreased significantly in T2 (4.2 ± 0.2 to 3.1 ± 0.3 g/dL; $p<0.01$), suggesting impaired protein synthesis or increased catabolism.

F. Female Hormonal Changes

Female fish exhibited a significant disruption of estrogenic pathways (Figure 2A). 17β-Estradiol (E2) decreased dose-dependently from 4.21 ± 0.22 ng/mL in controls to 1.45 ± 0.15 ng/mL in T2 (65.6% reduction; $p<0.001$). Conversely, testosterone (T) showed a non-monotonic response: slight increase in T1 (non-significant) followed by significant elevation in T2 (1.82 ± 0.15 to 2.92 ± 0.25 ng/mL; 60.4% increase; $p<0.01$). The E2/T ratio, a critical indicator of endocrine balance, decreased dramatically from 2.31 ± 0.18 to 0.50 ± 0.08 (78.4% reduction; $p<0.001$).

G. Male Hormonal Changes

Male fish displayed a distinct pattern of endocrine disruption (Figure 2B). Testosterone decreased significantly from 5.25 ± 0.28 to 3.05 ± 0.32 ng/mL (41.9% reduction; $p<0.001$). 11-Ketotestosterone (11-KT), the primary bioactive androgen in male teleosts, showed even greater suppression (8.52 ± 0.45 to 3.15 ± 0.40 ng/mL; 63.0% reduction; $p<0.001$). Paradoxically, 17β-estradiol increased in males from 0.28 ± 0.03 to 0.62 ± 0.05 ng/mL (121% increase; $p<0.01$), suggesting feminization through altered aromatase activity or impaired steroid clearance.

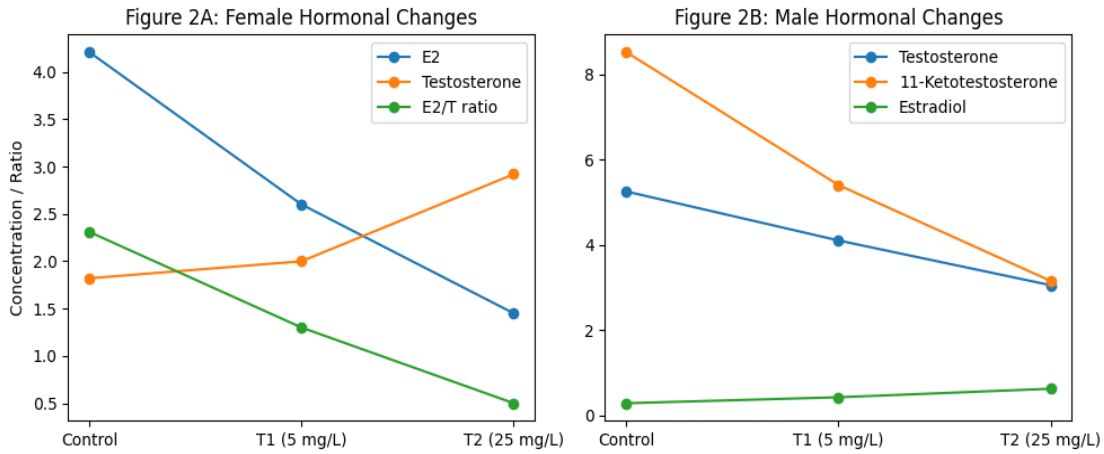

Figure 2: Dose-dependent alterations in sex steroid hormones in male and female *C. gariepinus*. (2A): Female Hormonal Changes (17β-Estradiol, Testosterone, E2/T Ratio). (2B): Male Hormonal Changes (Testosterone, 11-Ketotestosterone, 17β-Estradiol)

H. Lipid Peroxidation

Hepatic malondialdehyde (MDA) content, a marker of lipid peroxidation, increased dramatically with glyphosate exposure (Table 3). T2 fish exhibited 4.3-fold higher MDA levels compared to controls (5.12 ± 0.28 vs. 1.18 ± 0.08 nmol/mg protein; $p<0.001$), indicating severe oxidative membrane damage.

I. Antioxidant Enzyme Activities

Antioxidant defences were significantly modulated (Figure 3). Superoxide dismutase (SOD) activity increased 2.1-fold in T2 (52.7 ± 2.5 vs. 25.4 ± 1.2 U/mg protein; $p<0.001$). Catalase (CAT) showed similar induction (1.95-fold increase; $p<0.001$). This compensatory up regulation suggests activation of defence mechanisms against elevated reactive oxygen species (ROS).

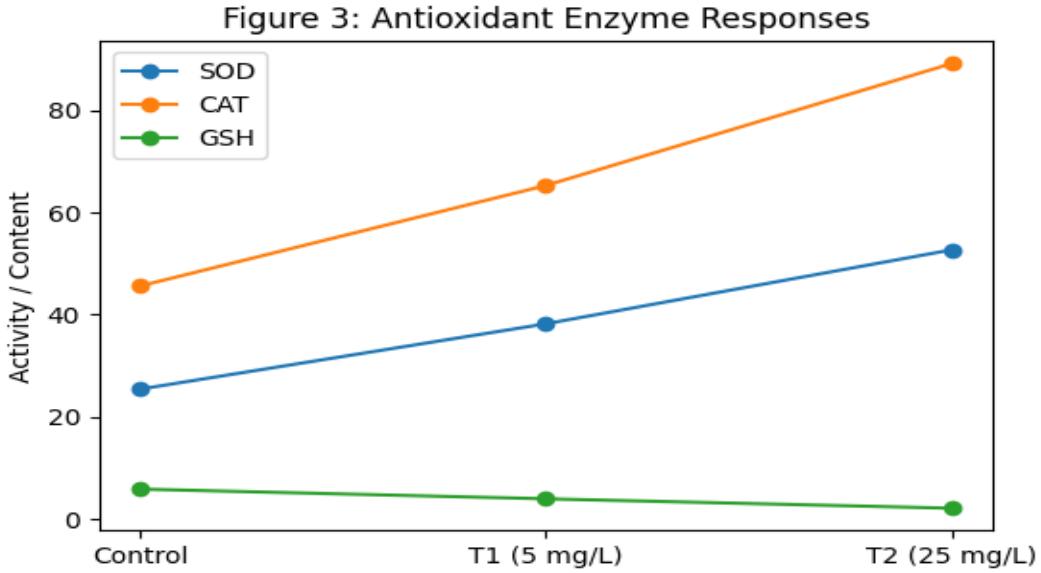

Figure 3: Dose-response relationships for SOD and CAT activities, with GSH content shown as complementary data oin *C. gariepinus* exposed to glyphosate for 60 days.

J. Reduced Glutathione (GSH) Depletion

Hepatic GSH content decreased dose-dependently, with T2 values 64.3% lower than controls (2.08 ± 0.18 vs. 5.82 ± 0.25 µmol/g tissue; $p<0.001$). This depletion of the primary non-enzymatic antioxidant indicates overwhelmed detoxification capacity and increased vulnerability to oxidative injury.

Table 3: Hepatic Oxidative Stress Parameters of *C. gariepinus* exposed to glyphosate for 60 days.

Parameter	Control	T1 (5 mg/L)	T2 (25 mg/L)	p-value
MDA (nmol/mg protein)	1.18 ± 0.08	2.85 ± 0.15**	5.12 ± 0.28**	<0.001
SOD (U/mg protein)	25.4 ± 1.2	38.2 ± 1.8**	52.7 ± 2.5**	<0.001
CAT (U/mg protein)	45.6 ± 1.8	65.3 ± 2.5**	89.2 ± 3.2**	<0.001
GSH (µmol/g tissue)	5.82 ± 0.25	3.92 ± 0.22*	2.08 ± 0.18**	<0.001

K. Organ somatic Indices

Gonad somatic Index (GSI) showed profound treatment effects with significant sex × treatment interaction ($F_{2, 84} = 12.45$, $p<0.001$; Figure 4). Female GSI decreased from 12.5 ± 0.5% in controls to 5.2 ± 0.3% in T2 (58.4% reduction; $p<0.001$). Male GSI showed parallel but less pronounced reduction (0.85 ± 0.04% to 0.41 ± 0.02%; 51.8% reduction; $p<0.001$). Leptosomatic Index (HSI) increased dose-dependently (1.82 ± 0.09% to 3.15 ± 0.18%; 73.1% increase; $p<0.001$), indicating possible hepatic hypertrophy or lipid accumulation.

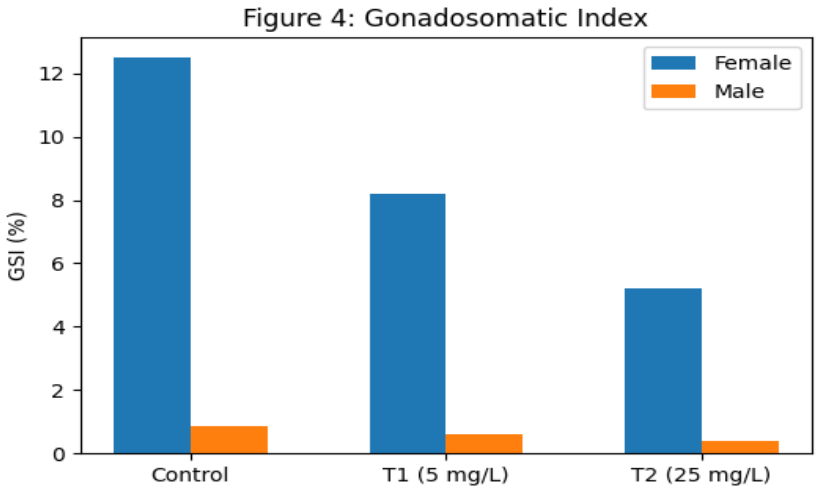

Figure 4: Comparing of GSI values for males and females across control, T1, and T2 treatments, highlighting sex × treatment interaction in *C. gariepinus* exposed to glyphosate for 60 days.

L. Sperm Quality Parameters

Sperm quality deteriorated dramatically with glyphosate exposure (Table 4). Total motility decreased from 84.8 ± 2.1% in controls to 27.8 ± 4.5% in T2 (67.2% reduction; $p<0.001$). Progressive motility showed even greater impairment (76.5 ± 2.5% to 15.2 ± 3.8%; 80.1% reduction). Sperm velocity parameters (VCL, VSL, and VAP) all decreased significantly. Sperm viability (membrane integrity) decreased from 92.3 ± 1.8% to 51.2 ± 5.2% in T2 ($p<0.001$). Sperm concentration was unaffected, suggesting qualitative rather than quantitative impairment.

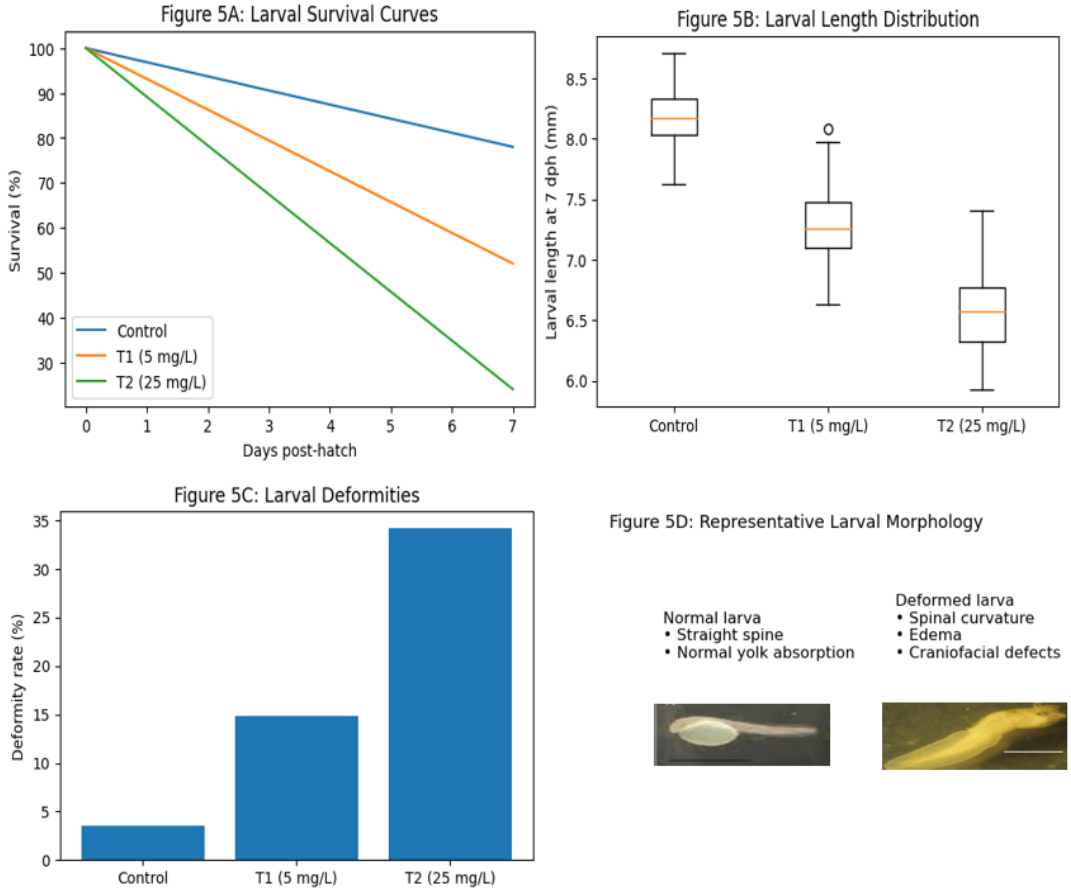

Figure 5: Trans generational effects on F1 generation of *C. gariepinus* exposed to glyphosate for 60 days (A) Larval survival curves, (B) Larval length distribution, (C) Deformity rates, and (D) Representative images of normal vs. deformed larvae.

M. Egg Quality and Fertilization

Egg diameter decreased dose-dependently (1.45 ± 0.03 mm to 1.18 ± 0.05 mm; 18.6% reduction; $p<0.001$). Percentages of irregularly shaped eggs increased from 5.2 ± 1.2% to 32.8 ± 4.5% ($p<0.001$). *In vitro* fertilization success showed severe impairment: fertilization rate at 4 hpf decreased from 87.8 ± 1.8% in controls to 31.8 ± 3.8% in T2

(63.8% reduction; p<0.001). Hatching success followed similar pattern (81.5 ± 2.2% to 24.5 ± 4.2%; 69.9% reduction; p<0.001).

Table 4: Gamete quality and fertilization parameters of *C. gariepinus* exposed to glyphosate for 60 days

Parameter	Control	T1 (5 mg/L)	T2 (25 mg/L)	p-value
Sperm Motility (%)	84.8 ± 2.1	61.5 ± 3.2**	27.8 ± 4.5**	<0.001
Progressive Motility (%)	76.5 ± 2.5	48.2 ± 3.8**	15.2 ± 3.8**	<0.001
Sperm Viability (%)	92.3 ± 1.8	78.2 ± 3.5*	51.2 ± 5.2**	<0.001
VCL (μm/s)	125.8 ± 8.5	88.5 ± 10.2*	45.2 ± 12.5**	<0.001
Egg Diameter (mm)	1.45 ± 0.03	1.32 ± 0.04*	1.18 ± 0.05**	<0.001
Irregular Eggs (%)	5.2 ± 1.2	15.8 ± 2.8*	32.8 ± 4.5**	<0.001
Fertilization Rate (%)	87.8 ± 1.8	64.5 ± 2.5**	31.8 ± 3.8**	<0.001
Hatchability (%)	81.5 ± 2.2	58.2 ± 3.0**	24.5 ± 4.2**	<0.001

N. Embryonic and Larval Development

Despite being raised in glyphosate-free water, offspring of exposed parents exhibited significant fitness impairments (Figure 6). Embryonic development was altered: time to 50% hatch increased from 28.5 ± 0.8 hours in control offspring to 42.2 ± 2.5 hours in T2 offspring (p<0.001). Larval survival at 7 dph decreased dose-dependently from 78.2 ± 2.5% to 23.8 ± 4.2% (69.6% reduction; p<0.001).

O. Larval Morphology and Deformities

Larval total length at 7 dph decreased from 8.2 ± 0.2 mm (control) to 6.5 ± 0.4 mm (T2; p<0.001). Yolk sac absorption was delayed (4.2 ± 0.2 days to 6.8 ± 0.4 days; p<0.001). Most strikingly, deformity rates increased dramatically: 3.5 ± 0.5% in controls vs. 34.2 ± 2.5% in T2 offspring (877% increase; p<0.001). Deformities included spinal curvature (lordosis, scoliosis), craniofacial abnormalities, pericardial edema, and fin malformations.

P. Larval Behaviour and Performance

Swimming performance assessed via escape response showed significant impairment: response latency increased from 0.42 ± 0.05 s in controls to 1.85 ± 0.25 s in T2 offspring (p<0.001). Swimming endurance (time to exhaustion in water current) decreased by 58.2% in T2 offspring (p<0.01).

Figure 6. Three-Dimensional PCA Showing Treatment Group Clustering

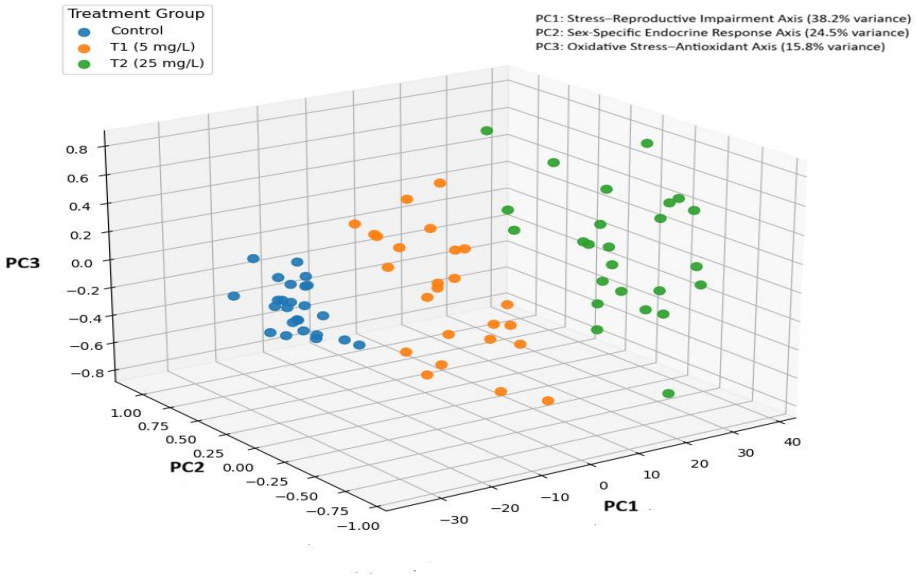

Figure 6: clustering of treatment groups along different principal components in *C. gariepinus* exposed to glyphosate for 60 days> PC1 (stress-reproductive axis) and PC2 (sex-specific axis).

Q. Multivariate and Dose-Response Analyses

PCA of 24 measured parameters explained 78.5% of total variance in the first three principal components (Figure 7). PC1 (38.2% variance) represented a "stress-reproductive impairment" axis, positively loaded by cortisol, MDA, atresia percentage, and negatively by GSI, E2, and fertilization rate. PC2 (24.5% variance) represented a "sex-specific response" axis, separating male and female hormone profiles. PC3 (15.8% variance) represented "oxidative stress-antioxidant" axis. Treatment groups clustered distinctly along PC1, with minimal overlap between control and T2.

R. Correlation Network Analysis

Strong correlations emerged among key endpoints (all $p<0.001$): Cortisol inversely correlated with GSI ($r = -0.82$), fertilization rate ($r = -0.80$), and larval survival ($r = -0.83$). MDA showed strong negative correlations with sperm motility ($r = -0.76$) and fertilization rate ($r = -0.79$). Parental exposure concentration correlated strongly with larval deformity rate ($r = +0.89$). Structural Equation Modeling suggested oxidative stress (MDA) as a primary mediator between glyphosate exposure and reproductive outcomes (standardized path coefficient = 0.68).

S. Dose-Response Modelling and ECx Values

Four-parameter logistic regression provided excellent fits for most endpoints ($R^2 > 0.90$; Table 5). Effective concentrations causing 10%, 25%, and 50% effect (EC_{10}, EC_{25}, EC_{50}) were calculated. Larval deformity showed greatest sensitivity (EC_{10} = 1.2 mg/L), followed

by sperm motility (EC_{10} = 1.8 mg/L) and fertilization rate (EC_{10} = 2.1 mg/L). The No Observed Effect Concentration (NOEC) was <5 mg/L for all reproductive endpoints; Lowest Observed Effect Concentration (LOEC) was 5 mg/L for most parameters.

Table 5: Dose-Response Parameters for Key Endpoints

Endpoint	Best-fit Model	R^2	EC_{10} (mg/L)	EC_{25} (mg/L)	EC_{50} (mg/L)
Fertilization Rate	Exponential decay	0.942	2.1	5.8	15.4
Sperm Motility	Linear	0.912	1.8	5.2	14.2
Female GSI	Exponential decay	0.963	1.5	4.5	12.8
Plasma Cortisol	Exponential growth	0.928	1.2	3.5	10.8
Hepatic MDA	Exponential growth	0.985	0.8	2.8	8.5
Larval Survival	Logistic decay	0.951	1.2	3.8	11.5
Larval Deformities	Logistic growth	0.972	0.9	2.5	7.8

IV. DISCUSSION

The present study highlights a coherent adverse outcome pathway (AOP) for glyphosate reproductive toxicity in *C. gariepinus* (Figure 8). Our integrated analysis suggests the following sequential events:

Glyphosate likely induces oxidative stress through multiple mechanisms:

[1] Disruption of mitochondrial electron transport chain, increasing ROS production (Peixoto, 2005);
[2] Depletion of GSH through conjugation or impaired synthesis (El-Shenawy, 2009);
[3] Interference with antioxidant enzyme activities. The 4.3-fold increase in hepatic MDA, coupled with GSH depletion and compensatory antioxidant up regulation, provides strong evidence for oxidative stress as a primary molecular insult.

The observed hormonal alterations suggest multiple endocrine targets. The decrease in female E2 coupled with increased T points to aromatase (CYP19) inhibition, consistent with *in vitro* studies showing glyphosate interference with aromatase activity (Gasnier *et al.,*2009). In males, the dramatic reduction in 11-KT, the primary androgen regulating spermatogenesis in teleost's (Borg, 1994), coupled with increased E2, suggests both impaired steroid genesis and altered steroid metabolism. Elevated cortisol, a potent inhibitor of gonadotropin release, likely suppresses the HPG axis at multiple levels (Pankhurst, 2011).

Sperm motility loss likely results from oxidative damage to sperm membranes and mitochondrial dysfunction (Aitken & Baker, 2013). The correlation between MDA and motility (r = -0.76) supports this mechanism. Reduced egg diameter and increased abnormalities suggest impaired vitellogenesis and oocyte maturation, consequences of disrupted estrogen signalling. The cascade culminates in severely reduced fertilization and

hatching success. The 64% reduction in fertilization rate at 25 mg/L represents reproductive failure with clear population-level implications.

Our findings reveal important sex differences in glyphosate toxicity. Females showed greater reduction in GSI (58% vs. 52% in males), suggesting higher sensitivity in ovarian tissue. This may relate to the energy-intensive nature of vitellogenesis and oocyte maturation, processes highly dependent on intact endocrine signalling and cellular energy metabolism. Conversely, males exhibited more severe gamete impairment (67% motility loss vs. 19% egg diameter reduction), possibly reflecting greater vulnerability of spermatozoa to oxidative damage due to high membrane polyunsaturated fatty acid content and limited antioxidant defences (Landsteiner *et al.,* 2010).

The opposing hormonal shifts-feminization in males (increased E2, decreased androgens) and masculinization in females (decreased E2, increased T)—suggest complex endocrine disruption beyond simple aromatase inhibition. Potential mechanisms include: altered expression of steroid genic enzymes (Star, CYP11, CYP17, and CYP19), changes in steroid receptor expression or binding, modified steroid clearance rates, or disruption of feedback loops in the HPG axis. These sex-specific responses have important implications for population dynamics, potentially altering sex ratios, breeding behaviours, and reproductive timing.

The strong dose-response relationships for oxidative stress markers, coupled with their correlations with reproductive endpoints, position oxidative stress as a central mechanism in glyphosate toxicity. MDA elevation indicates lipid peroxidation of cellular membranes, potentially affecting gonad structure, sperm membrane integrity, and oocyte quality. GSH depletion reduces capacity for xenobiotic detoxification and free radical scavenging, creating a vicious cycle of oxidative damage.

Antioxidant enzyme up regulation (SOD, CAT) represents a compensatory response, but one that may impose metabolic costs reflected in reduced growth and condition factor. The oxidative stress parameters showed excellent sensitivity (EC_{10} values 0.8-1.2 mg/L), suggesting their utility as early-warning biomarkers for glyphosate exposure in monitoring programs. The most concerning finding is the significant impairment in F1 generation despite no direct glyphosate exposure. Several mechanisms may contribute:

Parental exposure may induce heritable epigenetic changes affecting gene expression in offspring. DNA methylation alterations in germ cells could persist through fertilization, affecting embryonic development (Feil & Fraga, 2012). Oxidative stress can cause DNA damage in developing gametes, leading to mutations transmitted to offspring (Aitken & Baker, 2013).

Reduced egg quality (smaller size, altered composition) may limit resources available for embryonic development. Altered hormone levels in eggs could disrupt early developmental Programming. Poor sperm quality on the other hand may result in fertilization by suboptimal sperm, compromising embryo viability (Kime *et al.,* 2001). The high deformity rates (34.2% in T2 offspring) and reduced larval survival have clear population-level implications. If these effects occur in natural populations, they could reduce recruitment success and genetic diversity, with long-term consequences for population viability.

The EC_{50} values for reproductive endpoints (7.8-15.4 mg/L) are within the range of glyphosate concentrations reported in aquatic systems following agricultural runoff (up to 5-10 mg/L in edge-of-field waters, with pulses potentially higher; Battalion *et al.,* 2014). This suggests realistic risk of reproductive impairment in wild fish populations.

The NOEC of <5 mg/L and LOEC of 5 mg/L for most endpoints indicate that current water quality guidelines (typically based on acute toxicity or single endpoints) may be inadequately protective. Our multi-endpoint approach demonstrates that significant reproductive effects occur at concentrations well below those causing acute mortality.
Previous studies on glyphosate reproductive toxicity in fish have reported conflicting results, likely due to differences in species, exposure conditions, formulations, and endpoints examined.

The consistency of our findings across multiple biological levels strengthens confidence in the observed effects and their ecological relevance.

V. CONCLUSION

The result shows that chronic sub-lethal glyphosate exposure induces severe reproductive impairment in *Clarias gariepinus* through a cascade of physiological disturbances. Oxidative stress serves as a primary molecular initiating event, causing lipid peroxidation, antioxidant depletion, and cellular damage. Endocrine disruption manifests as sex-specific hormonal imbalances: feminization in males (reduced androgens, increased estragon) and masculinization in females (reduced estrogen, increased androgen), suggesting complex interference with steroid genesis, Gamete quality deterioration involves reduced sperm motility and viability, smaller egg size, and decreased fertilization competence, Trans generational effects occur even without direct offspring exposure, manifesting as reduced larval survival, increased deformities, and impaired development.

The adverse outcome pathway elucidated here-from molecular oxidative stress to population-relevant reproductive failure—highlights the ecological risk posed by glyphosate contamination in aquatic ecosystems. Effective concentrations for reproductive impairment (EC_{10} values 0.8-2.1 mg/L) fall within environmentally relevant concentrations, suggesting current regulatory thresholds may be inadequately protective. These findings underscore the necessity of incorporating sub-lethal reproductive endpoints and Trans generational assessments into ecological risk assessment frameworks for herbicides. Furthermore, they highlight the vulnerability of aquatic reproductive systems to chronic chemical stress, with implications for biodiversity conservation and sustainable fisheries management.

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