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Xeno-estrogenic metal-induced alterations in antioxidant enzyme activities, vitellogenin, and estrogen-receptor alpha in *Channa striata* brooders

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ABSTRACT

The study was conducted to evaluate the impact of effluents containing heavy metals on the water quality and physiological health of *Channa striata* brooders. It focused on changes in bioindices and biochemical biomarkers, comprising antioxidant enzyme activities (superoxide dismutase, catalase, and glutathione peroxidase) and metallothionein (MT) protein levels. Hormonal parameters, including testosterone and estradiol, along with the expression of vitellogenin (*Vtg*) and estrogen receptor alpha (*ERα*), were assessed in fish collected from two sites on the River Ganga, Narora and Kanpur, and a reference site, KGF at Aligarh. Kanpur (Jajmau) site receives effluents primarily from tannery industries and domestic sewage, while the Narora stretch is influenced by agricultural runoff as well as industrial discharges from the nearby lock-manufacturing units. Elevated concentrations of iron, nickel, chromium, cadmium, and lead at both sites due to contamination resulted in metal bioaccumulation in tissues (muscle, liver, and gonads). The liver showed the most pronounced site-specific variations in both sexes, including reductions in bioindices values and alterations in antioxidant enzyme activities, particularly at Kanpur. MT levels increased consistently from KGF to Kanpur (Jajmau) in both liver and gonads of brooders. Testosterone and estradiol levels declined in males and females, respectively, along the pollution gradient. In addition, site-specific expression patterns of *Vtg* and *ERα* genes revealed compromised reproductive health. Both genes were significantly upregulated, indicating metal-induced transcriptional activation.

1. INTRODUCTION

Anthropogenic activities and natural calamities tend to influence and degrade water resources across the globe [1]. Water from the Ganga River is used for several activities such as sustenance, agriculture, and industries [2]. Many cities and towns situated on the banks of the Ganga influence water quality, as wastes are often directly discharged into the river. Even when treatment plants are present, their efficiency is limited. In the Ganga basin, around 12,000 million L/day (MLD) of sewage is generated, while the treatment capacity is only about 5,000 MLD [3]. The contributions from municipal sewage and industrial effluents are approximately 70–80% and 15–20%, respectively [4-6]. In Kanpur city, which is one of the major urban and industrial centers along the Ganga, municipal solid waste generation has been estimated at approximately 1,400–1,500 tonnes/day [7], further exacerbating the pollution burden in this stretch of the river. These xenobiotics, especially heavy metals, influence aquatic life by disrupting physiological processes. There are several reports on parameters such

as temperature, dissolved oxygen (DO), total dissolved solids (TDS), pH, and conductivity influencing fish health [8,9], supporting the use of fish as bioindicators of water bodies. This is primarily due to the bioaccumulation of heavy metals in fish tissues, as they are non-biodegradable entities. Presence of trace and heavy metals in different tissues of fish has been observed by several workers [10,11]. The metals tend to influence several health conditions in fishes; however, this study intends to observe the effect on molecular, physiological, and biochemical responses in brooders. Bioindices such as hepatosomatic index (HSI), gonadosomatic index (GSI), and condition factor (K) are used for studying metabolic activities, reproductive and general health of fishes.

Heavy metals can affect various physiological and biochemical pathways, including those involving antioxidant enzymes [12]. In fish, enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) play a crucial role in maintaining normal cellular function by catalyzing reactions involving oxygen (O_2)-derived metabolites. SOD catalyzes the conversion of superoxide radicals into hydrogen peroxide (H_2O_2) and molecular O_2, while CAT facilitates the breakdown of H_2O_2 into water and O_2. GPx complements the actions of SOD and CAT by reducing H_2O_2 and organic hydroperoxides to water and alcohol, respectively. GPx utilizes reduced glutathione (GSH) as

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a co-substrate, converting it to oxidized glutathione (GSSG) in the process. GPx activity in fish can be affected by environmental factors, including the availability of selenium, which is essential for proper enzyme function [13].

Metallothionein's (MTs), low-molecular-weight, cysteine-rich proteins, play a fundamental role in metal homeostasis, detoxification, and antioxidant defense in fish [14]. MTs are frequently utilized as biomarkers for aquatic habitats polluted by heavy metals due to their strong affinity to metal ions [15]. The induction of MT synthesis in response to metal exposure is a well-documented defense mechanism in fish.

In addition to these biochemical markers, reproductive hormones play a pivotal role in assessing the reproductive health of fish under environmental stress. Testosterone, a key male reproductive hormone, is essential for spermatogenesis by promoting the proliferation and maturation of spermatogonia while sustaining normal testicular physiology [16]. Estradiol (17β-estradiol, E2), the most biologically active estrogen in fish, is critical for female reproductive function, promoting oocyte growth, ovarian follicle formation, and vitellogenesis [17]. Disruption in hormonal balance, particularly due to heavy metal exposure, can severely impair reproductive performance in fish populations. Vitellogenesis is the process by which the liver synthesizes vitellogenin (*Vtg*) under the stimulation of estrogen, and it is subsequently released into the bloodstream, transported to developing oocytes, and taken up via receptor-mediated endocytosis, where it forms the major yolk protein for embryo development [18]. Among various estrogen receptors in fish, estrogen receptor alpha (*ERα*) is considered the key mediator of estrogen action in *Vtg* synthesis. Its expression increases significantly during the breeding season and is strongly induced by exogenous estrogen treatment [19,20]. Typically, *Vtg* expression is upregulated only in adult females during gonadal maturation. However, the induction of *Vtg* and *ERα* in males is a strong indicator of estrogenic or heavy metal contamination, as these genes are not normally or minimally expressed in male fish [21,22]. Heavy metals can mimic estrogenic compounds, leading to the expression of these genes, making them reliable molecular biomarkers of endocrine disruption.

Channa striata, an indigenous species, was selected for this study due to its ecological significance and sensitivity to environmental changes. As a top predator in freshwater ecosystems, *C. striata* occupies a higher trophic level, making it more susceptible to biomagnification of toxicants, including heavy metals. In addition, *C. striata* exhibits more pronounced physiological responses to environmental stress, making it a more effective bioindicator [23]. The Ganga stretch at Kanpur and Narora offers a natural pollution gradient, allowing comparative assessment of heavy metal-induced physiological and biochemical alterations.

Previous studies have reported heavy metal pollution in the Ganga River and its effects on various physiological functions in fish [24,25]. However, there is limited understanding of how these metals affect endocrine function and molecular biomarkers in a sex-specific manner. So far, no comprehensive study has investigated *Vtg* and *ERα* expression in both males and females alongside hormonal profiling (testosterone, estradiol) across two stretches of the Ganga with varying pollution gradients. Therefore, it is hypothesized that heavy metal contamination in the Ganga leads to bioaccumulation in *C. striata*, thereby altering bioindices, antioxidant enzyme activities, MT levels, and inducing endocrine disruption that compromises reproductive health.

2. MATERIALS AND METHODS

2.1. Sample Collection and Analysis of Water Samples

Samples were collected from the Ganga River at Narora (28° 11′ 20.0" N 78° 23′ 48.0" E) and Kanpur (26° 26′ 14.4" N 80° 24′ 12.6" E) during the monsoon season. Sites were selected based on various sources of pollution. Being an industrial center, Kanpur receives a large amount of untreated wastewater from textile mills, tanneries, and other industries. Narora is a relatively less industrialized region, although agricultural activities are prevalent. KGF Hatchery (27° 49′ 22.1" N 77° 56′ 50.8" E) was selected as a reference site, which is an important aquaculture facility contributing to fish breeding and seed production.

A total of 60 fishes were collected across all study sites using drag nets. After capture, the live fishes were carefully transported to the laboratory and acclimatized for several hours to reduce stress before further experimental processing.

2.1.1. Analysis of physicochemical parameters and heavy metal estimation of water samples

TDS, conductivity, and pH were measured using electronic meters (HANNA), while transparency was measured on the spot by Secchi disk. Temperature was measured using a thermometer (Deluxe, 6). Water was fixed at the spot and brought to the laboratory to determine DO by the Winkler iodometric method [26]. Heavy metals, including Cu, Fe, Ni, Cr, Cd, and Pb, were estimated in water as per the standard protocol of APHA [27] using an Atomic Absorption spectrophotometer (AAS, AAS PinAAcle 900F, Perkin Elmer).

2.2. Heavy Metal Estimation in Fish Tissues

Ten specimens were collected from each site and dissected in the laboratory for heavy metal estimation in the liver, gonads, and muscle tissues. No disinfectants were used during dissection, as the aim was to study the tissues under natural environmental conditions without introducing chemical alterations. A total of 60 fish ($n = 60$) were collected across all study sites. From each site, 20 fishes were collected, including 10 males and 10 females ($n = 10$). The tissues were oven-dried at 55–60°C for 5–6 h and powdered using a mortar and pestle. A 2-gram sample from each powdered tissue was digested with HNO_3 and $HClO_4$ in a 4:1 ratio in borosilicate beakers. The beakers were placed on a hot plate at 40–45°C until a clear, transparent solution was obtained, cooled, and filtered to remove any undissolved impurities. The digested samples were then brought to a final volume of 50 ml using volumetric flasks and mixed thoroughly for heavy metal estimation. The concentrations of Cu, Fe, Ni, Cr, Cd, and Pb in tissues were determined using an atomic absorption spectrophotometer (AAS PinAAcle 900 F) following the standard protocol of APHA [27].

2.3. Bioindices Measurement

The total length of fish was measured using a stainless-steel ruler, and its weight was measured using a weighing balance (KERRO BL-P3/5002). The length and weight of the captured fish were recorded. They were euthanized by pithing to dissect the liver and gonads to calculate bioindices such as HSI, condition factor (K), and GSI as proposed by Desai [28] using the formulae:

HSI = Weight of the liver (g)/total fish weight (g) × 100

K = Total fish weight (g)/length^3 (cm) × 100

GSI = Weight of the gonads (g)/total fish weight (g) × 100

2.4. Biochemical Assays

2.4.1. Homogenate preparation

Fragments of gonads and liver were stored at −80°C until homogenization. For MT content analysis, tissue samples were homogenized in Tris-HCl/saccharide buffer (20 mM/500 mM, pH 8.6), and for the analysis of other biochemical biomarkers, gonadal and hepatic tissue were homogenized in potassium phosphate buffer (0.1 M, pH 7.0). After homogenization, samples were centrifuged at 15,000 × g for 30 min at 4°C. Aliquots of the supernatant for each biomarker were stored at −80°C.

2.4.2. Antioxidant enzyme activities

2.4.2.1. Estimation of SOD

SOD activity was assayed according to the protocol of McCord and Fridovich [29]. During the measurement process, the reaction mixture (3 mL) consisted of sodium phosphate buffer (50 mmol/L, pH 7.8), methionine (13 mmol/L), nitroblue tetrazolium (75 μmol/L), riboflavin (2 μmol/L), and 50 μL of the tissue supernatant. The reaction mixture was incubated for 15 min in fluorescent light under conditions of 25°C. Meanwhile, a UV/Vis spectrophotometer (UV-Helios 5 ALPHA MODEL) was used to measure the absorbance at 560 nm. Non-illuminated mixtures without enzyme extract were treated as control. The enzyme volume that corresponded with 50% inhibition of the reaction was estimated.

A blank was first prepared under fluorescent light without tissue supernatant, and the percent inhibition of the reaction was calculated using the formula:

$$\text{Percent inhibition} = ([\text{Absorbance of control} - \text{Absorbance of sample}]/\text{Absorbance of control}) \times 100$$

The enzyme volume that produced 50% inhibition of the superoxide-driven reaction was identified based on this calculation. One unit (U) of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reaction under the assay conditions. The final enzyme activity was normalized to the total protein content and expressed as U/mg protein.

2.4.2.2. Estimation of CAT

CAT activity was estimated following the method of Lushchak and Bagnyukova [30]. The reaction mixture consisted of 50 mM potassium phosphate (KPi) buffer (pH 7.0), 0.5 mM EDTA, 10 mM H_2O_2, and 5–50 μL of tissue supernatant. CAT activity was measured at 240 nm using a UV–visible spectrophotometer (UV-Helios 5 Alpha model).

2.4.2.3. Estimation of GPx

GPx activity was determined following the method of Paglia and Valentine [31]. Briefly, a reaction solution was prepared in 20 mL of phosphate buffer (100 mM; pH 7.0) containing EDTA 5 mM, 3.12 g of NADPH, 10 μL of glutathione reductase (500 U/mg of protein/mL), 250 μL of NaN3 100 mM, and 7.68 g of reduced glutathione. Then, 510 μL of the reaction solution, 30 μL of milli-Q water, and 30 μL of tissue supernatant were added to the optical cell. The enzymatic reaction was initiated with the addition of 60 μL of 4 mM H_2O_2. The decrease in absorbance due to NADPH oxidation to $NADP^+$ was monitored at 340 nm for 5 min using a UV–visible spectrophotometer (UV-Helios 5 Alpha model).

2.4.3. MT concentration

The concentration of MT protein in liver and gonads was analyzed by the method of Viarengo *et al.* [32] using ethanol, chloroform, HCl, Tris-HCl buffer (20 mM), NaCl (0.25 M), sodium EDTA (4 mM EDTA and 1.0 M HCl), and Ellman's solution (DTNB; Methanol, and 0.2 M sodium phosphate buffer saturated with 2.0 M NaCl). The concentration of reduced sulfhydryl was estimated with a UV spectrophotometer (UV-Helios 5 Alpha model) by taking the absorbance at 412 nm. For quantification, a standard calibration curve was prepared using reduced glutathione (GSH) in the concentration range of 0–500 μg/mL. The standard curve exhibited high linearity, with a coefficient of determination ($R^2 = 0.9933$), indicating strong reliability of the assay. The concentration of MT in the samples was calculated using GSH as a standard, considering that 20 mol of cysteine is present in 1 mol of MT.

2.5. Hormonal Assay

The size of *C. striata* used in the study ranged from 32.0 to 36.7 cm across different sampling sites, and approximately 1.5–2.5 mL of blood was collected from each specimen. Blood was drawn from the caudal vein of live fish using a syringe and allowed to rest at room temperature for 1 h. The samples were then centrifuged (Agile, CentriteQ) at 1,000–2,000 × g for 10 min to obtain the serum, which was immediately transferred to a clean polypropylene tube using a Pasteur pipette. Hormonal analysis (testosterone and estradiol) was carried out using the Elecsys kit (Roche Diagnostics Ltd., India).

2.6. Genotoxicity Studies

Total RNA from liver was extracted using Trizol (Sigma) by vigorously shaking it and centrifuging in acidic conditions. The upper aqueous phase was collected, treated with an equal volume of isopropyl alcohol, and incubated at −20°C for 30 min. The mixture was then centrifuged at 10,000 RPM at 4°C for 10 min. After centrifugation, the supernatant was discarded and the collected pellet was dried, and then nuclease-free water was added to it [33]. The obtained RNA integrity was checked using agarose gel electrophoresis. For gel preparation, 1 g of agarose was added to 100 mL of 1X TAE (Tris-Acetate EDTA) buffer to prepare a 1% agarose gel. Out of this, 50 mL of molten gel was poured into a casting tray (Medox Biotech India Pvt. Ltd.) containing 2 μL of ethidium bromide and allowed to solidify for 20 min with a comb in place. After solidification, 2 μL of 6X DNA loading dye and 10 μL of RNA sample were loaded into each well. Electrophoresis was carried out at 110 V for 45–60 min, and the bands were visualized under UV light. The Orcinol method was used for quantification of RNA [34]. dT column was used for the isolation of mRNA. For cDNA synthesis by reverse transcription, 4ug of each sample was used using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, Cat. No. K1621) in a thermocycler (MWG Biotech). The synthesized cDNA was used for quantification of *Vtg*, *ERα,* and the β-actin genes using real time quantitative PCR (RT-qPCR, AB-Applied Biosystem, 7000 system software). The primers (forward and reverse) were designed using Primer Express 3.0, as indicated in Table 1. Melting curve analysis was performed over a range of 45–95°C to study the target amplification specificity. 7000 System Software was used to obtain threshold cycle (Ct) values. Corresponding Ct values of β-actin were used to normalize each Ct value of the target gene. The 2-ΔΔCt method was used to determine the relative expression levels of target genes [35].

2.7. Ethical Statement

All the experiments adhered to the guidelines prescribed by the Ministry of Environment and Forests, Government of India, under registration no. CPCSEA/BC/P-8/2023-24 issued by the Committee for Control and Supervision of Experiments on Animals, New Delhi, and

approved by the Institutional Animal Ethical Committee, Department of Biochemistry, Aligarh Muslim University, Aligarh, India.

2.8. Statistical Analysis

In the present study, each experiment was conducted with three independent replicates ($n = 3$), and each replicate included samples from ten individuals ($n = 10$). Before analysis, data were tested for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene's test. Statistical comparisons among different sites were performed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. In addition, for parameters in which male and female data were presented together (antioxidant enzyme activities and gene expression profiles), a two-way ANOVA, followed by Tukey's *post hoc* test, was applied. Pearson's correlation coefficient (r) was used to assess the relationship between heavy metal concentrations and MT levels across different tissues. All statistical analyses were carried out using GraphPad Prism software (version 8.4.3.686), and the results were expressed as mean ± standard deviation (SD). Differences were considered statistically significant at $P < 0.05$.

3. RESULTS

3.1. Physicochemical Parameters and Heavy Metal Analysis at Selected Sites

3.1.1. Physicochemical parameters of water

The physicochemical parameters of the water samples from the three study sites, KGF Pond (reference site), Ganga River at Narora, and Kanpur (Jajmau), differed significantly ($P < 0.05$), as summarized in Table 2.

Water temperature remained within the ideal range (20–30°C) at KGF and Narora (26.5°C and 28.5°C, respectively), except at Kanpur, which showed an increase in temperature above the ideal range. Water at KGF had an ideal amount of DO (7.67 mg/L), which declined at Narora **(**5.58 mg/L) and Kanpur (3.38 mg/L**)**. Desired (168 mg/L) TDS values were observed at the reference site, while higher values at Narora (482.33 mg/L) and Kanpur (585 mg/L) surpassed the permissible threshold. Water at KGF was slightly alkaline (7.9), while Narora had a slightly lower but acceptable pH of 7.2. In contrast, the pH (6.16) at Kanpur was acidic. EC at all the sites was within the acceptable range (100–1000 μS/cm). Water transparency was below ideal values at all the sites.

3.1.2. Heavy metal concentration in water

Heavy metal analysis across the three study sites revealed a significant ($P < 0.05$) increase in pollution levels at the Ganga River, with KGF Pond (reference site) showing minimal pollution [Table 2]. At KGF Pond, only Fe was detected at a concentration of 0.185 mg/L, within the permissible range (0.3–1.0 mg/L), indicating an uncontaminated environment. In contrast, heavy metals at both sites, except Cu, exceeded the recommended limits. The order of heavy metal concentration at Narora was Fe > Ni > Cr > Cd > Pb > Cu, while at Kanpur, it was Fe > Cr > Pb > Cd > Ni > Cu.

3.2. Heavy Metal Bioaccumulation in the Tissues

The analysis of heavy metal accumulation in both brooders collected from Kanpur and Narora revealed significant ($P < 0.05$) differences in the levels of Cu, Fe, Ni, Cr, Cd, and Pb across different tissues and sites, with Kanpur showing mostly higher levels due to greater industrial activities, particularly tanneries.

As depicted in Tables 3 and 4, heavy metal accumulation in different tissues of both male and female brooders is presented. The order of accumulation was similar in both sexes, showing the highest

Table 1: Forward and reverse primer sequences used for RT-qPCR analysis.

Gene	Forward	Reverse	Product bps	Accession Number
B-actin	GGTATGTGCAAGCCGGATTC	CCAGATCTTCTCCATGTCATC	173	JF423931
ERα	GGATGGGAGGAAACAGTAG	TCTCATCCGTGTTGGTCAGC	197	GU969584.1
Vtg	TATGAGGCGTTGCTCCTTGG	CCAGGGCTAAAGTCAACTTG	202	MK112905.1

Table 2: Description of physicochemical properties of the study sites and heavy metal concentration of the water samples.

Parameters	KGF Pond, Aligarh*	Ganga River Narora	Ganga River, Kanpur (Jajmau)	Ideal Conditions (CPCB, 2019) & (WHO 2004/USEPA, (Metals)
Temperature (°C)	26.5±0.95[c]	28.5±1.32[b]	30.73±0.66[a]	20-30
Dissolved oxygen (mg/L)	7.67±0.52[a]	5.58±0.25[b]	3.38±0.45[c]	>6.0
Total dissolved solids (mg/L)	168±11.13[c]	482.33±10[b]	585±17[a]	500
pH	7.9±0.2[a]	7.2±0.26[b]	6.16±0.30[c]	6.5-8.5
Conductivity (μS/cm)	72±5.29[c]	322.66±4.04[b]	504±8[a]	1000
Transparency (m)	0.533±0.02[a]	0.36±0.03[b]	0.3±0.02[c]	1.5-3.5
Cd	Bdl	0.235±0.004[b]	0.376±0.003[a]	0.003 mg/L
Ni	Bdl	0.383±0.003[a]	0.304±0.004[b]	0.02 mg/L
Cu	Bdl	0.103±0.001[b]	0.185±0.003[a]	2/1.3 mg/L
Cr	Bdl	0.369±0.002[b]	0.711±0.002[a]	0.05/0.1 mg/L
Fe	0.185±0.001[c]	1.554±0.026[b]	2.666±0.012[a]	0.3-1.0 mg/L
Pb	Bdl	0.182±0.002[b]	0.404±0.003[a]	0.05 mg/L

Values given as mean±SD. ANOVA was used to test the significance of means. Means with different superscripts in a row indicates a significant difference set at a significance level of *P*<0.05. Guidelines from CPCB: Central Pollution Control Board, 2019, and heavy metal permissible limits by WHO, 2004 and USEPA). ∗ Indicates reference site. Bdl: below detection limit

Table 3: Bioaccumulation of heavy metals (Cu, Fe, Ni, Cr, Cd & Pb) in different tissues (liver, testes and muscle) of male across the study sites.

Organ	Sites	Cu	Fe	Ni	Cr	Cd	Pb
Liver	KGF*	bdl	40.35±0.49c	bdl	bdl	bdl	bdl
	Narora	B60.74±1.03b	A150.76±0.67b	C40.39±0.97b	D33.22±0.59b	F26.41±0.56b	E32.78±1.57b
	Kanpur	B66.3±0.94a	A166.63±1.23a	D42.01±0.78a	C47.74±0.92a	F29.04±0.67a	E39.07±1.53a
Testes	KGF*	bdl	25.26±0.62c	bdl	bdl	bdl	bdl
	Narora	C18.33±0.49b	A46.37±1.08b	B23.03±0.76a	D16.51±0.58b	CD17.42±0.52b	E9.39±0.82b
	Kanpur	B22.14±1.19a	A50.89±1.75a	BC21.36±1.29b	B23.02±1.73a	C20.68±1.02a	D15.22±0.53a
Muscles	KGF*	bdl	3.24±0.36c	bdl	bdl	bdl	bdl
	Narora	B18.22±0.45b	A38.05±0.78b	C17.35±0.53a	D13.35±0.48b	E11.21±0.46b	D13.28±0.49b
	Kanpur	C20.31±0.75a	A47.0±1.19a	D16.09±1.05b	B24.54±1.16a	D15.44±0.70a	C19.22±0.81a
	FAO	10	5.6	0.5-0.6	1	1	0.05

Values given as mean±SD. ANOVA was used to test the significance of means. The different superscripts (capital, row: HM accumulation in a tissue) (small column: HM accumulation comparing the sites) indicate a statistically significant difference set at a significance level of $P<0.05$. * Indicates the reference site, and bdl is below the detection limit. Concentration of heavy metals is in mg/Kg dw

Table 4: Bioaccumulation of heavy metals (Cu, Fe, Ni, Cr, Cd & Pb) in different tissues (liver, ovaries and muscle) of female across the study sites.

Organ	Sites	Cu	Fe	Ni	Cr	Cd	Pb
Liver	KGF*	bdl	46.01±0.13c	bdl	bdl	bdl	bdl
	Narora	BC40.21±1.44b	A153.21±3.14b	C39.64±0.98a	B41.07±0.82a	E21.35±1.46b	D30.82±0.74b
	Kanpur	B53.30±0.77a	A170.67±1.1a	D35.26±0.75b	E30.33±0.50b	F25.19±1.12a	C37.01±0.8a
Ovaries	KGF*	bdl	33.21±1.21c	bdl	bdl	bdl	bdl
	Narora	CD19.84±0.90b	A54.36±0.90b	B29.33±1.17b	F16.79±1.16b	E18.19±1.24b	DE19.31±1.17b
	Kanpur	C28.67±0.49a	A66.41±0.94a	B33.67±0.41a	F23.54±0.46a	E24.04±0.80a	DE24.31±0.72a
Muscles	KGF*	bdl	1.31±0.23c	bdl	bdl	bdl	bdl
	Narora	F10.80±1.09b	A32.44±1.37b	B22.38±0.83a	E12.25±0.92b	D14.31±0.93b	C17.30±1.49a
	Kanpur	B21.73±0.49a	A41.49±0.58a	D11.76±0.50b	C16.96±1.00a	B20.62±0.69a	D10.60±0.58b
	FAO	10	5.6	0.5-0.6	1	1	0.05

Values given as mean±SD. ANOVA was used to test the significance of means. The different superscripts (capital, row: HM accumulation in a tissue) (small column: HM accumulation comparing the sites) indicate a statistically significant difference set at a significance level of $P<0.05$. * Indicates the reference site, and bdl is below the detection limit. Concentration of heavy metals is in mg/Kg dw

bioaccumulation in the liver, followed by the testes, with muscles displaying the lowest concentrations. In males, Pb bioaccumulated more in muscles than in testes. The order of heavy metal accumulation in the liver at Kanpur was Fe > Cu > Cr > Ni > Pb > Cd. At Narora, a similar trend was observed in the liver, except for Ni, which exceeded Cr levels. In the testes, the bioaccumulation sequence at Kanpur was Fe > Cr > Cu > Ni> Cd> Pb, while a different order was observed at Narora, where the pattern was Fe > Ni > Cu > Cd> Cr > Pb. Muscle tissues at both sites contained metal concentrations exceeding the permissible limits established by FAO and WHO. The bioaccumulation pattern in muscle tissue at Kanpur was Fe > Cr > Cu > Pb> Ni > Cd, whereas at Narora, the pattern followed the order was Fe > Cu > Ni > Cr > Pb > Cd. In females, the pattern of bioaccumulation in the liver at Kanpur followed the order Fe > Cu > Pb> Ni > Cr > Cd, whereas the bioaccumulation at Narora sites was Fe > Cr > Cu > Ni> Pb> Cd. In contrast, the bioaccumulation patterns in the ovaries differed from those in the testes, where concentrations and the order of accumulation were Fe > Ni > Cu > Pb > Cd > Cr. In addition, muscle tissue exhibited varying accumulation patterns at both sites in females, with higher concentrations of heavy metals recorded at Kanpur, except for Ni. The accumulation order for muscle tissue is Fe > Cu > Cd > Cr > Ni > Pb at Kanpur, whereas at Narora, it is Fe > Ni > Pb > Cd> Cr> Cu. Notably, heavy metal concentrations in muscle tissues exceeded the permissible limits established by the FAO and WHO.

Table 5: Bio indices in male and female across the sites.

Gender	Sites	Gonadosomatic index (GSI)	Hepatosomatic index (HSI)	Condition factor (K)
Male	KGF*	0.292±0.001a	1.122±0.002c	1.025±0.003a
	Narora	0.238±0.002b	1.138±0.002b	0.933±0.002b
	Kanpur	0.212±0.003c	1.17±0.007a	0.784±0.003c
Female	KGF*	5.95±0.025a	0.959±0.006a	1.124±0.011a
	Narora	5.35±0.042b	0.863±0.003b	1.009±0.012b
	Kanpur	4.75±0.048c	0.779±0.007c	0.937±0.003c

Values given as mean±SD. ANOVA was used to test the significance of means. The different superscripts in a column indicate a statistically significant difference set at a significance level of $P<0.05$ across three sites. * Indicates reference site

3.3. Effect of Heavy Metals on Bioindices

Heavy metals had a significant effect on both males' and females' bioindices, which are summarized in Table 5. Comparative analysis across gender and sites indicated that female brooders significantly exhibited higher GSI, demonstrating increased reproductive investment. Among sites, the highest GSI values were observed at KGF, while Kanpur recorded considerably lower GSI values for both genders. On comparing HSI across sites and genders, males displayed the highest HSI values, especially at Kanpur. The condition factor (K), which measures general health and robustness, was higher in females than males across all
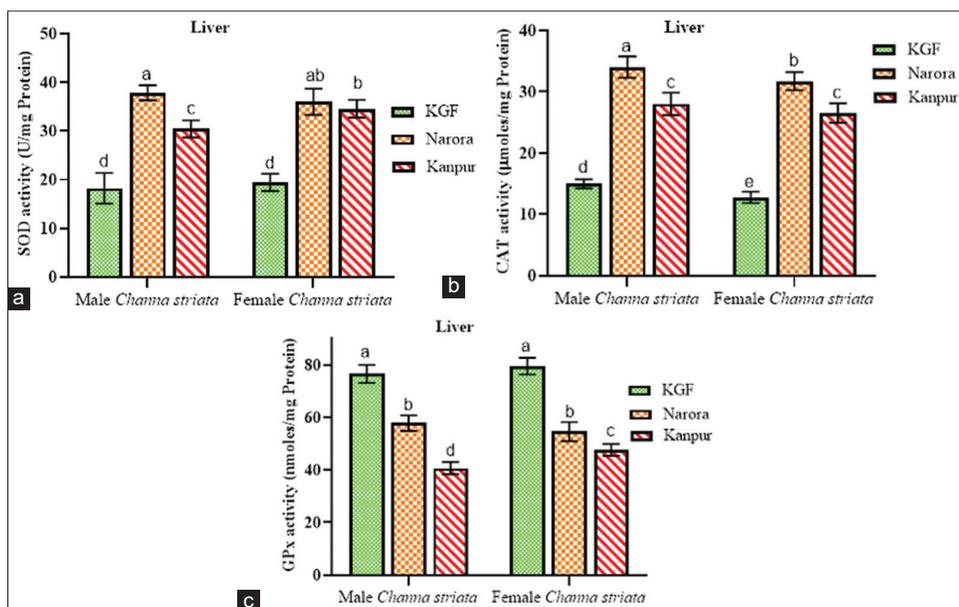
Figure 1: (a-c) Superoxide dismutase, catalase, and glutathione peroxidase activity in the liver of male and female *Channa striata* collected from reference and study sites. Data are presented as mean ± standard deviation. Statistical comparisons were performed using two-way analysis of variance followed by Tukey's *post hoc* test. Different superscript indicates a statistically significant difference set at a significance level of $P < 0.05$.
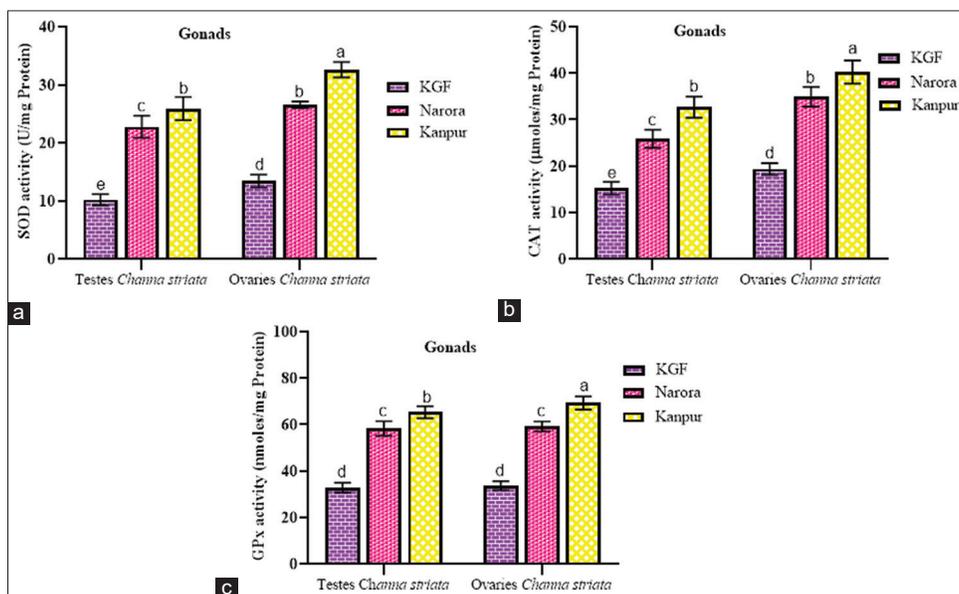

Figure 2: (a-c) Superoxide dismutase, catalase, and glutathione peroxidase activity in the gonads of male and female *Channa striata* collected from reference and study sites. Data are presented as mean ± standard deviation. Statistical comparisons were performed using two-way analysis of variance followed by Tukey's *post hoc* test. Different superscript indicates a statistically significant difference set at a significance level of $P < 0.05$.

sites. This is consistent with their larger gonads and greater investment in reproduction, which increases the body condition factor.

3.4. Effect of Heavy Metals on Antioxidant Enzyme Activities in Liver and Gonads

The effect of heavy metals on antioxidant enzymes (SOD, CAT, and GPx) in the liver and gonads of male and female brooders is depicted in Figures 1 and 2, analyzed using two-way ANOVA.

3.4.1. Effect of heavy metals on antioxidant enzyme activities in the liver

SOD and CAT activities were significantly higher ($P < 0.05$) at Narora and Kanpur compared to the reference site (KGF) in both sexes. For both enzymes, activities were consistently lower at Kanpur than at Narora, with males exhibiting a greater reduction than females. In contrast, GPx activity in liver tissues showed a significant ($P < 0.05$) decrease at both Narora and Kanpur compared to KGF in male and female brooders.

3.4.2. Effect of heavy metals on antioxidant enzyme activities in the gonads

The activities of SOD, CAT, and GPx in the gonadal tissues (testes and ovaries) exhibited a significant ($P < 0.05$) increase at both sites in comparison to the reference site. For all enzymes, activities were consistently greater at Kanpur than at Narora in both sexes. Among the tissues, the ovaries displayed significantly ($P < 0.05$) higher SOD
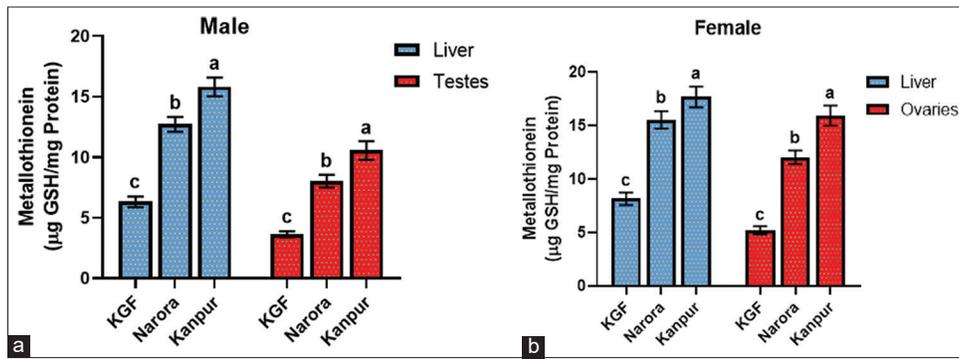
Figure 3: (a and b) Metallothionein content in the liver and gonads of male and female *Channa striata* collected from reference and study sites. Data are presented as mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance. Different superscript indicates a statistically significant difference set at a significance level of $P < 0.05$.
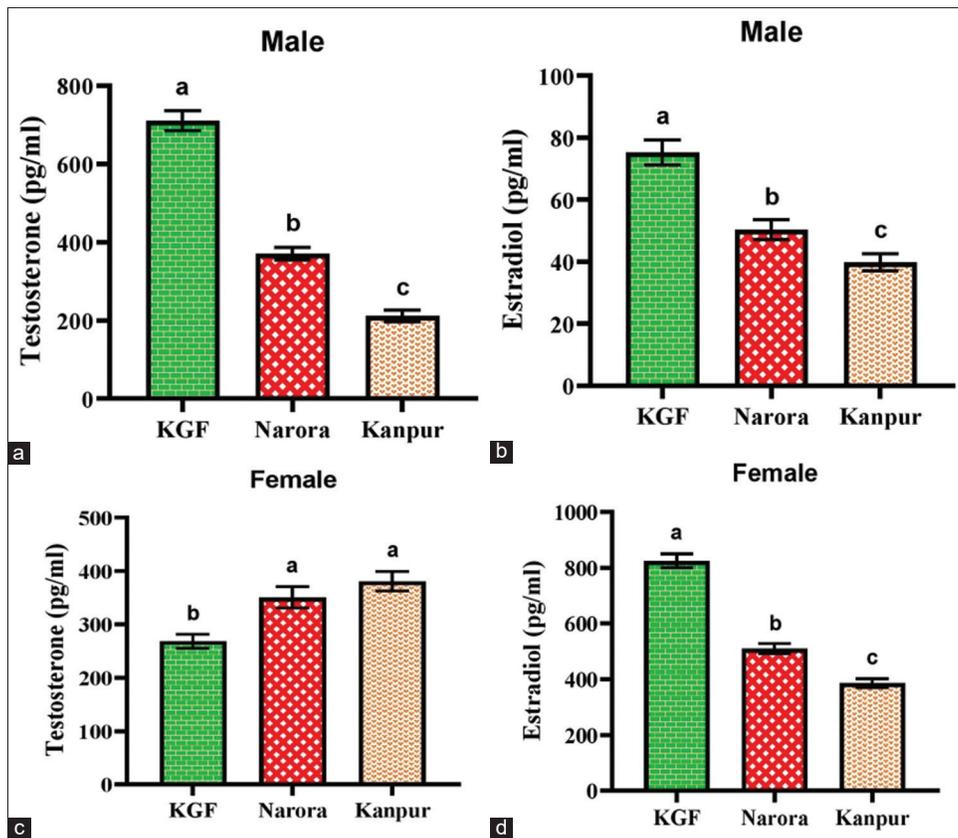

Figure 4: (a and b) Testosterone and estradiol in male *Channa striata,* whereas (c and d) testosterone and estradiol in female *Channa striata* from all three sites. Data are presented as mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance. Different superscripts indicate a statistically significant difference set at a significance level of $P < 0.05$.

and CAT activities than testes at both Narora and Kanpur, suggesting a sex-specific variation in enzyme response.

A similar pattern was observed for GPx activity, where enzyme levels increased at the contaminated sites in both sexes, with the elevation being more pronounced in the ovaries than testes at Kanpur.

3.5. MT Concentration in the Liver and Gonads

MT levels in the liver and gonads of brooders across the three sites are presented in Figure 3. The MT content in the liver and gonads (testes and ovaries) showed a significant ($P < 0.05$) increase at both contaminated

sites. In males, both the liver and testes exhibited elevated levels of MT at the Narora and Kanpur sites. Similarly, females showed a consistent increase in MT content in both the liver and ovaries across the study sites. The increase in MT was more pronounced in the gonadal tissues, particularly in the ovaries, than in the liver.

3.6. Effect of Heavy Metals on Reproductive Hormones

A significant ($P < 0.05$) elevation in reproductive hormone levels along different levels of environmental contamination was noted [Figure 4]. In males, testosterone concentrations demonstrated a marked decline corresponding to the increasing pollution levels. The highest testosterone
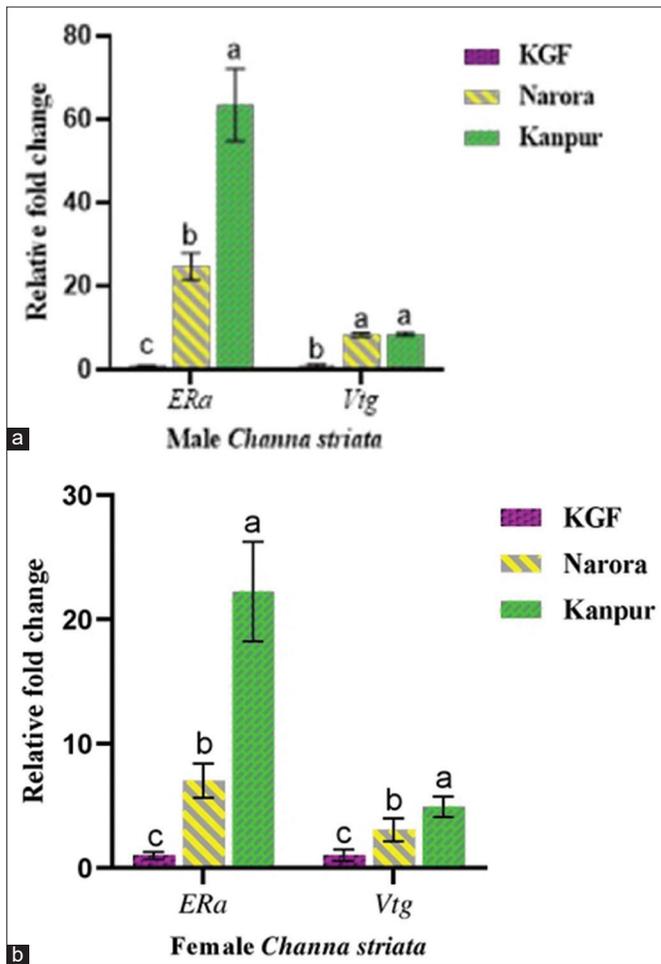
Figure 5: (a and b) The relative expression of estrogen receptor-alpha (*ERα*) and Vitellogenin (*Vtg*) in *Channa striata* based on sites. Data are presented as mean ± standard deviations. Statistical analysis was performed by one-way analysis of variance. The different superscripts indicate a statistically significant difference set at a significance level of $P < 0.05$.

Figure 6: (a and b) The relative expression of vitellogenin (*Vtg*) and estrogen receptor-alpha (*ERα*) in *Channa striata* based on gender across reference and study sites. Data are presented as mean ± standard deviation. Statistical comparisons were performed using two-way analysis of variance followed by Tukey's *post hoc* test. Different superscript indicates a statistically significant difference set at a significance level of $P < 0.05$.

level was observed at the KGF reference site, followed by a substantial reduction at Narora, with the lowest level at Kanpur. Estradiol levels in males exhibited a similar decreasing trend, indicating suppression of gonadal steroidogenesis under elevated heavy metal stress. In females, estradiol concentrations declined significantly ($P < 0.05$) across the sites. This decline is consistent with pollution-induced impairment of ovarian function. Testosterone levels in females significantly increased at the polluted sites compared to the reference site.

3.7. *Vtg* and *ERα* as Genotoxic Biomarkers of Metal Toxicity

3.7.1. Site-specific expression of Vtg and ERα
The expression levels of *ERα* and *Vtg* genes were analyzed and compared among sites to assess the molecular effects of heavy metal contamination [Figure 5]. *ERα* expression levels exhibited a marked increase in both male and female fish collected from the Ganga river sites, indicating a clear response to heavy metal pollution. In males, *ERα* expression was significantly ($P < 0.05$) elevated at Kanpur (63.49) and Narora (24.86) when compared to the reference site. Similarly, in females, *ERα* expression was significantly ($P < 0.05$) higher at Kanpur (22.21) and Narora (7.03) relative to KGF. *Vtg* gene expression levels were also elevated at the contaminated sites. In males, *Vtg* expression
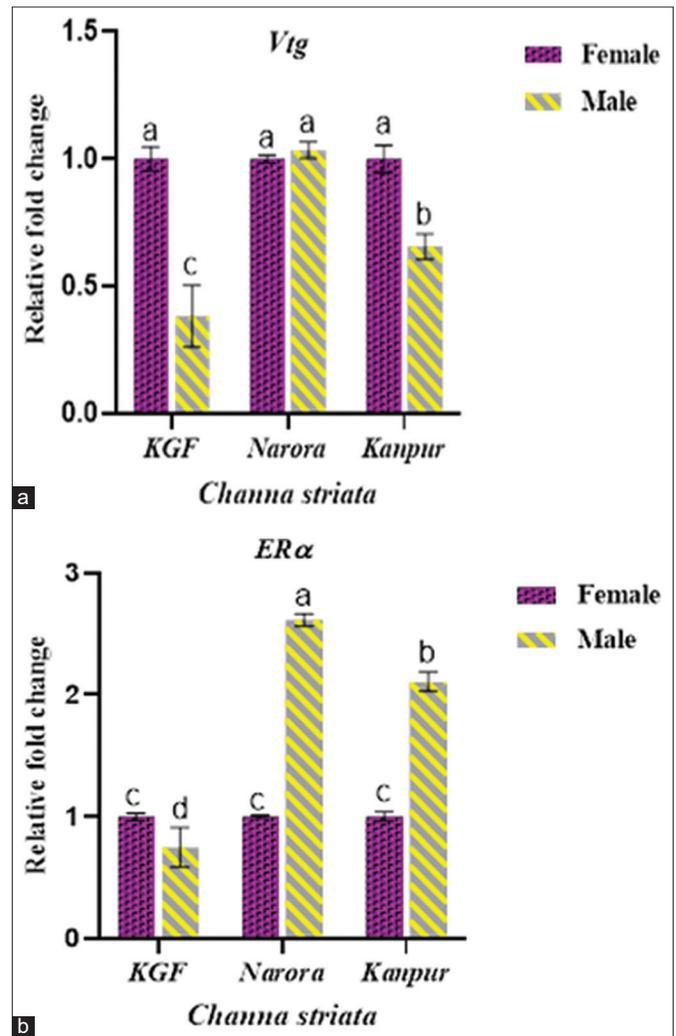
showed a significant ($P < 0.05$) increase at Kanpur (8.55) and Narora (8.40) relative to the KGF. In females, expression followed the same trend, with significantly ($P < 0.05$) higher levels at Kanpur (4.93) and Narora (3.09) compared to KGF.

3.7.2. Sex-specific expression of Vtg and ERα
Both *Vtg* and *ERα* demonstrated sex-specific differences depicted in Figure 6, analyzed by two-way ANOVA. At the reference site (KGF), males exhibited markedly lower *Vtg* and *ERα* expression than females. Male *Vtg* levels increased at both contaminated sites; at Narora, they reached values comparable to females, whereas at Kanpur, male *Vtg* expression remained significantly ($P < 0.05$) lower than females. *ERα* expression in males was significantly lower at KGF but increased markedly at both Narora and Kanpur. At Narora, males exhibited the highest *ERα* expression, which was significantly ($P < 0.05$) greater than the corresponding female group. At Kanpur, males also showed significantly ($P < 0.05$) higher *ERα* levels than females, although the magnitude of induction was lower than at Narora.

Table 6: Correlation between heavy metals (Cu, Fe, Ni, Cr, Cd, and Pb) and metallothionein levels in the liver and testes of male *Channa striata*.

Heavy metals	Liver		Testes	
	P-value	Pearson value (r)	*P*-value	Pearson value (r)
Cu	<0.001	0.958	<0.001	0.960
Fe	<0.001	0.967	<0.001	0.962
Ni	<0.001	0.946	<0.001	0.892
Cr	<0.001	0.987	<0.001	0.971
Cd	<0.001	0.957	<0.001	0.955
Pb	<0.001	0.971	<0.001	0.982

All correlations were significant at *P*<0.001.

Table 7: Correlation between heavy metals (Cu, Fe, Ni, Cr, Cd, and Pb) and metallothionein levels in the liver and ovaries of female *Channa striata*.

Heavy metals	Liver		Ovaries	
	P-value	Pearson value (r)	*P*-value	Pearson value (r)
Cu	<0.001	0.981	<0.001	0.984
Fe	<0.001	0.979	<0.001	0.985
Ni	<0.001	0.931	<0.001	0.960
Cr	<0.001	0.876	<0.001	0.980
Cd	<0.001	0.973	<0.001	0.977
Pb	<0.001	0.980	<0.001	0.972

All correlations were significant at *P*<0.001.

3.8. Correlation Analysis between Heavy Metals and MT

MT was correlated with heavy metals because it shows a direct dose-dependent response to metal exposure, whereas other physiological markers are influenced by multiple regulatory factors.

3.8.1. Correlation analysis between heavy metals and MT in male *C. striata* liver

Correlation analysis showed that MT levels in the male *C. striata* liver were positively and significantly correlated with all the heavy metals analyzed [Table 6]. The correlation coefficients were $r = 0.958$ ($P < 0.001$) for Cu, r = 0.967 ($P < 0.001$) for Fe, r = 0.946 ($P < 0.001$) for Ni, r = 0.987 ($P < 0.001$) for Cr, r = 0.957 ($P < 0.001$) for Cd, and r = 0.971 ($P < 0.001$) for Pb. These results indicate that MT concentrations increased consistently with increasing levels of heavy metals in the livers of male *C. striata*.

3.8.2. Correlation analysis between heavy metals and MT in female *C. striata* liver

In the female liver, MT levels also showed significant positive correlations with all the heavy metals (Table 7, $P < 0.001$). The correlation coefficients were r = 0.981 ($P < 0.001$) for Cu, r = 0.980 ($P < 0.001$) for Pb, r = 0.979 ($P < 0.001$) for Fe, r = 0.973 ($P < 0.001$) for Cd, r = 0.931 ($P < 0.001$) for Ni, and r = 0.876 ($P < 0.001$) for Cr. These findings confirm that MT levels increased proportionately with rising metal accumulation in the female *C. striata* liver.

3.8.3. Correlation analysis between heavy metals and MT in male *C. striata* testes

Correlation analysis showed that MT levels in the testes of male *C. striata* were positively and significantly correlated with all the heavy metals studied (Table 6, $P < 0.001$). The correlation coefficients were r = 0.960 ($P < 0.001$) for Cu, r = 0.962 ($P < 0.001$) for Fe, r = 0.892 ($P < 0.001$) for Ni, r = 0.971 ($P < 0.001$) for Cr, r = 0.955 ($P <$ 0.001) for Cd, and r = 0.982 ($P < 0.001$) for Pb. These results indicate that MT levels in the male testes increased consistently with increasing heavy metal accumulation.

3.8.4. Correlation analysis between heavy metals and MT in female *C. striata* ovaries

Correlation analysis showed that MT levels in the ovaries of female *C. striata* were positively and significantly correlated with all the heavy metals studied (Table 7, $P < 0.001$). The correlation coefficients were r = 0.984 ($P < 0.001$) for Cu, r = 0.985 ($P < 0.001$) for Fe, r = 0.960 ($P < 0.001$) for Ni, r = 0.980 ($P < 0.001$) for Cr, r = 0.977 ($P < 0.001$) for Cd, and r = 0.972 ($P < 0.001$) for Pb. These results indicate that MT levels in the female ovaries increased correspondingly with increasing concentrations of heavy metals.

4. DISCUSSION

The observed variations in water quality parameters among the three sites indicate distinct pollution levels, with Kanpur showing maximum deviation from ideal conditions due to intensive anthropogenic activities and tannery effluent discharge. Elevated temperature, reduced DO, higher TDS, and conductivity at Kanpur reflect organic and inorganic loadings that increase microbial O_2 demand. Tannery effluents rich in chromium, lead, cadmium, and organic matter elevate BOD, further depleting DO [36]. In addition, untreated sewage and agricultural runoff contribute fertilizers and salts, raising conductivity and turbidity [37]. These findings agree with reports highlighting the Kanpur to Varanasi stretch as a hotspot of anthropogenic stress [38]. Among heavy metals, Fe and Cr were most abundant due to tannery discharge and industrial runoff [39]. Metals such as Pb, Cd, and Ni are linked to dyeing and electroplating in leather processing, while Cu and Ni are used for antimicrobial and finishing purposes [40,41]. Geological weathering also contributes Fe through lithological leaching in the Ganga basin [42]. Higher Ni levels at Narora likely originate from local lock industries. Agricultural inputs further raise Cd, Pb, and Cr concentrations, especially during monsoon runoff [43]. Thus, the metal contamination pattern across sites reflects both industrial and agricultural sources. Higher metal accumulation in liver than muscle supports its role as the primary detoxification organ, capable of metal sequestration through metalloproteins like ferritin [44-46]. In contrast, muscle tissues accumulate fewer metals due to lower metabolic activity and limited metal-binding sites.

Higher GSI in females reflects their greater reproductive investment during the breeding season. The reduced GSI values at Kanpur suggest that heavy metal pollution interferes with hormonal regulation and diverts energy away from gonadal development, leading to suppressed reproductive investment. Similar reductions in GSI under metal exposure have been reported in a study [47]. Elevated HSI values in males at Kanpur indicate severe hepatic stress due to elevated heavy metal bioaccumulation, which can cause hepatocellular injury and fatty degeneration [48]. In contrast, females showed lower HSI values, likely because hepatic reserves were mobilized for gonadal development during the spawning phase, resulting in high GSI but reduced liver weight [49]. The condition factor (K) was higher in females due to larger gonads, and overall K values were highest at KGF, reflecting better environmental conditions. The lowest K values at Kanpur suggest impaired health and poor condition of *C. striata* under heavy metal pollution.

At the polluted sites, variations in heavy metal concentrations were associated with changes in antioxidant enzyme activities in the

gonads, suggesting altered cellular regulation under contaminated conditions [50]. However, in the liver, although SOD and CAT activities increased, GPx activity declined, a pattern that may reflect differences in enzyme sensitivity or regulation under higher heavy metal exposure [51]. Similarly, a study observed a decrease in GPx in Indian major carps exposed to heavy metal exposure, which was related to a decline in GSH and glutathione reductase (GRd) activity [52]. These patterns suggest that heavy metal exposure may affect hepatic antioxidant enzyme functioning, particularly at Kanpur. The greater reduction in GPx in males further points to sex-related differences in enzyme responsiveness, with males appearing more susceptible under heavy pollution. This male-specific susceptibility might be due to physiological or hormonal differences influencing metal accumulation and antioxidant enzyme regulation [53,54]. In contrast, females tended to exhibit relatively higher antioxidant activities in the gonads, possibly reflecting reproductive-associated metabolic demands during oocyte development [55].

The elevation of MT observed across tissues and genders reflects a physiological defense mechanism against heavy metal exposure. MT binds and sequesters metals such as Cu, Cd, Pb, and Cr, thereby reducing their toxic effects. A study also found that exposure to heavy metals in *Prochilodus costatus* increased MT levels [56]. The progressive increase in MT from KGF to Narora and Kanpur corresponds with the rising metal burden along this pollution gradient, suggesting its usefulness as an indicator of metal exposure. Although the liver is the primary detoxification organ and accumulates higher metal concentrations, comparatively greater MT induction in the ovary can be attributed to reproductive physiology. During the breeding season, MT concentrations in the liver tend to decrease to release Zn, which is required to stabilize membrane structures involved in *Vtg* synthesis and secretion [57,58]. As a result, ovarian MT levels remain elevated, which may contribute to maintaining metal homeostasis within the gonads and support reproductive processes under contaminated conditions. The positive correlations observed between heavy metals and MT levels across liver and gonadal tissues further support this association. The strong correlation indicates that MT synthesis increased in direct proportion to metal burden, confirming that MT induction is a metal-responsive regulatory mechanism rather than a random physiological variation.

The hormone variations across sites indicate heavy metal-induced endocrine disruption. The decrease in testosterone in males at Narora and Kanpur is linked to metal interference with steroidogenic enzymes and the hypothalamic–pituitary–gonadal axis, consistent with findings from the Kor River, where fish exposed to lead (Pb) and cadmium (Cd) exhibited lower estradiol and testosterone levels compared to those from uncontaminated regions [59]. Metals can inhibit 17β-hydroxysteroid dehydrogenase, directly reducing testosterone biosynthesis [60]. In females, the decline in estradiol may be attributed either to the toxic effects of metals on the hypothalamic–pituitary axis or to impaired estradiol production by germinal cells [61,62]. The elevated testosterone in females suggests aromatase (CYP19A1a) inhibition by metals such as Pb and Cr, preventing conversion of testosterone to estradiol [63]. The strong association between Pb, Cd, and Cr bioaccumulation in gonadal tissues and altered hormone levels aligns with previous studies on other fish species, where similar patterns of hormonal disruption and metal bioaccumulation were observed [64,65].

The increased expression of *ERα* and *Vtg* at Narora and Kanpur indicates estrogenic pathway activation in response to heavy metal exposure. Several heavy metals are known to act as xenoestrogens or interfere with estrogen signaling, which explains the induction of these genes under polluted conditions [22,66]. Since *ERα* and *Vtg* synthesis are normally regulated by 17β-estradiol (E2), their expression in males is widely recognized as a biomarker of endocrine disruption [21]. The greater induction of *ERα* in males may reflect higher metal accumulation and greater sensitivity, possibly due to sex-specific differences in metal uptake and detoxification processes [53]. The low expression at the reference site (KGF) further confirms that heavy metals are responsible for this response. The induction of *Vtg* in males also highlights that male fish can serve as effective indicators of estrogenic pollution, as they possess the necessary receptors and cellular machinery for *Vtg* synthesis [67].

5. CONCLUSION

The study confirms that heavy metal contamination in the Ganga River causes bioaccumulation in *C. striata*, leading to altered antioxidant enzyme activities, hormonal imbalance, and bioindices. Elevated MT levels and changes in *Vtg* and estrogen receptor alpha expression reflect severe endocrine disruption at polluted sites, particularly Kanpur. These findings validate the hypothesis that heavy metal exposure impairs reproductive health and establish *C. striata* as a reliable bioindicator of aquatic metal toxicity.

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors requirements/guidelines.

7. ACKNOWLEDGMENT

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

All experimental procedures were conducted in accordance with the guidelines prescribed by the Ministry of Environment and Forests, Government of India, under registration no. CPCSEA/BC/P-8/2023–24, issued by the Committee for Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, and were approved by the Institutional Animal Ethics Committee, Department of Biochemistry, Aligarh Muslim University, Aligarh, India.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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