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Research Article

Paternal exposure to environmentally relevant levels of di-(2-ethylhexyl) phthalate (DEHP) disrupts breeding success and offspring immune health in koi carp (*Cyprinus carpio*)

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Abstract: Di-(2-ethylhexyl) phthalate (DEHP), a common plasticizer regarded as prevalent environmental pollutant. Although, the reproductive toxicity of DEHP in aquatic animals are well documented, but its impact on offspring immunity after parental exposure remains unknown. In this study, mature male koi carp were exposed to DEHP at three distinct environmentally relevant levels (1, 10 and 100 µg/L) for 150 days. Pairing of exposed males with untreated females at post-exposure significantly lowered the fertilization, hatching and larval survival rates. Further, the larvae of each group were reared separately up to 40 days in control condition to evaluate the transgenerational immune effects of DEHP. The length gain, weight gain and specific growth rate were reduced significantly in the fingerlings of exposed groups. Histological investigations revealed the cellular structural impairment in liver and kidney tissues of the paternally treated fingerlings. The levels of immune and stress-response biomarkers, such as HSP70, HSP90, IgM and cortisol were fluctuated in the fingerlings of exposed groups. The mRNA study of immune-related genes (*IL1β*, *IL10*, *IL8*, *TNFα*, *TLR5*, *iNOS*, *TGFβ*, *C3*, *NF-κB* and *MyD88*) exhibited differential expression patterns in the paternally impacted fingerlings. Findings of this study reveals that DEHP exposure not only affect the exposed individual, yet also the future generations.

Key words: *Cyprinus carpio* (koi carp); Di-(2-ethylhexyl) phthalate; Breeding performances; Immune and stress-response biomarkers; Immune gene expressions

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1. Introduction

Di-(2-ethylhexyl) phthalate often called DEHP is extensively utilized as a plasticizer, primarily found in the polyvinyl chloride (PVC) plastics, medical devices and various consumer products [1, 2]. Globally, DEHP is produced in quantities of 3 to 4 million tons each year, representing 50% of the total plasticizer production [3, 4]. DEHP does not form chemical bonds with polymers and typically leaks out as a liquid, contaminating the surrounding environment and posing a risk to those nearby [5, 6]. Despite the prevalence, DEHP has been categorized to be an endocrine-disrupting chemical (EDC), which means that it may interfere with the hormonal systems of living organisms, including aquatic species [7, 8]. As a result, DEHP has emerged as an environmental contaminant of concern, particularly in aquatic ecosystems, where it could be released into water bodies via industrial discharge, waste and consumer products. DEHP levels in polluted regions can vary from 98 to 218 µg/L, whereas drinking water and surface water have the concentrations of 1 and 10 µg/L, respectively [9, 10]. In Indian river systems, DEHP has been detected at notable levels with maximum concentrations of 24.46 µg/L in the Kaveri, 13.43 µg/L in the Vellar and 2.77 µg/L in the Thamiraparani [11]. Even more alarming concentrations have been reported elsewhere, such as in the Ogun river, Nigeria in which DEHP levels reached as high as 480 µg/L [12]. Fish come into contact with DEHP by consuming contaminated food and through direct exposure to polluted water, silt or clay [13, 14].

Fish as an essential component of aquatic ecosystems are particularly vulnerable to the effects of chemical contaminants like DEHP. Considering the increasing number of evidences demonstrate that DEHP negatively impacts reproductive health, while parental effects on the immune system of their offspring is an area of emerging concern in case of fishes. Investigations have indicated that exposure to DEHP may cause reproductive impairments in male fish, affecting on spermatogenesis, sex hormone production, fertility and overall breeding success [15-18]. Exposure to environmental chemicals can have effects that are passed down to future generations through paternal transmission mechanisms, such as altering sperm epigenetics, inducing germline mutations and modifying seminal fluid composition [19, 20]. Paternal exposure has

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been shown to cause transgenerational effects in offspring, which may include developmental abnormalities, immune system dysfunction and altered behavior [21-23]. This highlights the potential long-term consequences of environmental contamination on fish populations, as the capability for recovery from such exposure may be compromised by reduced fertility, impaired immune responses and poor survival rates in subsequent generations.

Assessing the impacts of environmentally relevant DEHP concentrations on koi carp (*C. carpio*) breeding performances and how these effects extend to their offspring was the aim of the present investigation. In the market, koi carp are the most popular ornamental fish because of their numerous varieties of color patterns as well as breeding ease [24]. Besides, this fish can extremely tolerate to variations in water quality, stocking density and grows quickly [25]. For these types of physio-toxicological studies, this extensively farmed species serves as an excellent biological model due to gonadal development is activated in small size [26, 27]. In this study, male koi carps were subjected to three distinct concentrations of DEHP (1, 10 and 100 μg/L) for 150 days, a period encompassing gonadal development and the reproductive phase. This chronic exposure allows assessment of cumulative effects on spermatogenesis, endocrine regulation and germline integrity, which are critical for evaluating paternal-mediated transgenerational outcomes. After the exposure duration, exposed males were paired with untreated females and potential effects on spawning performances were assessed. Further, the larvae of different experimental groups were reared up to 40 days in control condition to evaluate their health status. The immune parameters of fingerlings were investigated through the assessment of histology (liver and kidney), immune and stress-response biomarkers as well as mRNA expression of immune genes. Additionally, GC-MS technique has been used to detect the presence of DEHP in exposed males and their offspring. The outcomes of this study offer important insights to evaluate the possible effects of DEHP on aquatic creatures.

2. Materials and Methods

2.1. Chemicals and experimental fish

Analytical-grade di-(2-ethylhexyl) phthalate (DEHP) along with acetone were obtained from the SRL Pvt. Ltd., India. To prepare the stock solution, 100 g/L of acetone was used to dissolve DEHP, which was then stored at 4°C until needed. The concentrations of exposure were prepared by serially diluting the stock solution as required. Furthermore, all of the chemicals used in this investigation were of the technical grade.

The male and female mature koi carp (*C. carpio*) weighed an average of 42.4 ± 2.24 g and measured a total length of 15.2 ± 1.15 cm were obtained from a local fish dealer. For 20 days, the fish were acclimated in a cement cistern (3000 L) at the outdoor facility of Fisheries Sciences Department, Vidyasagar University, West Bengal, India. Once acclimated, the male fish were divided randomly to different groups for exposure study. However, the female fish were reared in the same tank without any chemical exposure.

2.2. Exposure experiment

Four separate groups were set up: three groups received varying concentrations of DEHP along with one control group to assess the impact of DEHP on koi carp breeding performances. Twelve adult males were kept in each of the twelve glass tanks (a group comprised of three tanks) with water capacity of 180 L. Three groups received DEHP exposure: one group received 1 μg/L of water (as a lower concentration), another group received 10 μg/L of water (as a medium concentration) and a third group received 100 μg/L of water (as a higher concentration). For comparison, a control group devoid of DEHP was also kept. For every group, including the control group, solutions with a solvent of 0.001% (v/v) acetone were provided. The water within each experimental tank had been changed once a week and the stock solution was used to prepare the assigned DEHP concentrations for exposure of fish. Carp feed (Cargill Pvt. Ltd., India) was provided to the experimental fish twice a day for 150 days at a body weight of 4%. During the exposure period, the physico-chemical parameters of water, such as temperature varied between 30 to 32°C, while the alkalinity, pH and dissolved oxygen were 88.5 ± 4.68 mg/L, 7.6 ± 0.64 and 5.4 ± 0.42 mg/L, respectively. With a photoperiod containing 12 h light and 12 h dark, the exposure study was carried out in an aerated condition.

2.3. Breeding performances

After the completion of chronic exposure period, twelve concrete spawning tanks with water capacity of 250 L (three tanks for a group) were used to keep four untreated females along with males (n = 4) from the glass tanks of each DEHP-treated group and control group. WOVA-FH injections (Biostadt India Ltd., India) were administered to the untreated females at a dosage of 0.2 mL/kg body weight. DEHP-exposed as well as control males were similarly injected with the same inducing substance at a dosage of 0.1 mL/kg body weight. A nylon net with mesh size of 0.5 mm was fastened to the bottom of each spawning tank to hold the sticky koi carp egg. Following the six hours of hormone injection, ovulation took place and the brooders were taken out of the spawning tank. Hatching occurred after the 30-35 h of spawning and the new larvae started exogenous feeding after 72 h post-fertilization. Hatched larvae were initially fed with commercial microparticulate diet (Cargill Pvt. Ltd., India) for one week. The following formulas were used to determine the rates of fertilization, hatching as well as survival of larvae.

$$\text{Fertilization rate (\%)} = \frac{\text{Fertilized eggs in number}}{\text{Total ovulated eggs in number}} \times 100$$

$$\text{Hatching rate (\%)} = \frac{\text{Hatchlings in number}}{\text{Total fertilized eggs in number}} \times 100$$

$$\text{Survival rate (\%)} = \frac{\text{Survived larvae in number}}{\text{Total hatchlings in number}} \times 100$$

2.4. Post larval rearing and growth parameters

After measuring the initial length and weight, fifty number of post larvae (10 days old) from each group were transferred to new aquarium (180 L) without any exposure. They were maintained for additional 30 days in triplicate with proper aerated condition until became fingerlings. The water quality parameters were similar as mentioned in exposure experiment section. To fulfill the nutritional requirements, live feed, i.e., *Artemia* was provided as a supplementary feed during this time. On the final day, the length and weight of fingerlings from each group were determined and calculated the growth parameters like length gain, weight gain as well as specific growth rate.

2.5. Ethical approval

Regarding the handling and utilization of lab animals, the guidelines of Institutional Animal Ethics Committee of Central Inland Fisheries Research Institute (IAEC/2021/04), West Bengal, India were followed strictly when conducting the experiment. Every process was designed with the goal of minimizing fish suffering as much as possible.

2.6. Sampling for analysis

Three males from each group (DEHP-exposed groups along with control group) were anesthetized for euthanasia after the 150 days of exposure period through the use of tricaine methanesulfonate (MS-222). They were sacrificed and testes were collected to find out whether DEHP accumulation occurred. Furthermore, the 40 days old fingerlings of control and DEHP-treated groups were anesthetized by using the same substrate. Afterwards, five fingerlings from each group were euthanized, followed by careful excision of the liver and kidney. These samples were preserved separately in Bouin's fixative as well as RNAlater (kept at −20°C) for histological study and RNA extraction purpose, respectively. However, five fingerlings were chopped into small pieces utilizing sharp sterile blade and made a single pool to quantify the DEHP concentrations. To estimate the immune and stress-response biomarkers, blood was drawn from the caudal fin base of five fingerlings from each group using a syringe and moved to a 1 mL centrifuge tube free of anticoagulant agent and kept for 20–25 min at normal temperature. Clotted blood samples were subsequently centrifuged at 6000 × *g* for 8 min at 4°C. Finally, the serum was gently pipetted into screw-cap tubes and stored at −20°C for further analysis.

2.7. Quantification of DEHP

Accumulated DEHP concentrations were measured from testes of control and DEHP-exposed males as well as in their offspring (fingerlings). Selective ion monitoring (SIM) and electron impact mode were implemented to measure the DEHP concentrations using a gas chromatography-mass spectrometer (ISQ 7000 GC–MS, Thermo Scientific, USA) equipped with a 30 m × 0.25 mm × 0.25 μm capillary column (TG-35MS Column, Thermo Scientific, USA). A brief description about the preparation of tissue samples and the GC-MS conditions may be found in the supplemental file.

2.8. Histological analysis

After 24 h fixation in Bouin's solution, the liver and kidney tissues were dehydrated in different gradients of ethanol, followed by xylene treatment (as a clearing agent). The treated tissues were embedded in paraffin wax and subsequently sectioned with thickness of 5 μm utilizing a rotating microtome (RM 2025, Leica Biosystems, Germany). After being stained with hematoxylin and eosin (H & E) stain, the sectioned tissues were examined for cellular alternations under a light microscope (Axio Lab A1, Carl Zeiss, Germany). A total of 6 slides per tissue were examined from fingerlings of each paternally exposed group. Histological changes were scored semi-quantitatively based on the average number of lesions observed in each slide and classified as none (–, no lesions), mild (+, 1–4 lesions), moderate (++, 5–8 lesions) or severe (+++, 9–10 lesions) following Shirdel et al. [28].

2.9. Immune and stress-response biomarkers

The serum samples were taken out from −20°C and allowed to reach room temperature. Additionally, the samples were inverted carefully and centrifuged again at 4000 × *g* for 4 min in cold condition. The immunoglobulin M (IgM), cortisol and heat shock proteins (HSP70 and HSP90) levels in serum samples were determined using commercial ELISA kits (EA0008FI, EA0004FI, EA0011FI and E0064FI, respectively) obtained from BT Lab Bioassay Technology, China. Following the manufacturer's instruction, all the assays were performed. Finally, a microplate reader (iMark 1681130, Bio-Rad, USA) was employed to take the OD at 450 nm.

2.10. RNA extraction and synthesis of cDNA

For the extraction of total RNA from the preserved tissue (liver and kidney), TRIzol (Sigma-Aldrich, Merck Group, USA) reagent was applied following the recommendations provided by the manufacturer. Prior to being treated with DNAse I (Turbo DNA-free kit, Thermo Scientific, USA), the RNA concentrations in every sample was measured employing a Nano-drop (Eppendorf AG 22331, Germany). A 1% agarose-containing gel was then utilized to assess the quality of each RNA sample. A first strand cDNA synthesis kit (K1622, Thermo Scientific, USA) was subsequently applied to process all RNA samples in accordance with the manufacturer's directions to synthesize cDNA. Before being used again, the samples of cDNA were stored at −20°C. As a housekeeping gene, the *18S rRNA* was employed for amplification of every cDNA sample. Lastly, a Thermal Cycler Gene Amp PCR system 9700 (Applied Biosystems, USA) was used to amplify all the target genes, which included interleukin 1 beta (*IL1β*), interleukin 10 (*IL10*), interleukin 8 (*IL8*), tumor necrosis factor alpha (*TNFα*), toll-like receptors 5 (*TLR5*), inducible nitric oxide synthase (*iNOS*), transforming growth factor beta (*TGFβ*), complement factor 3 (*C3*), nuclear factor-κB (*NF-κB*) and myeloid differentiation primary response 88 (*MyD88*) (illustrate briefly in the supplementary file).

2.11. Quantitative polymerase chain reaction (qPCR)

To perform the quantitative PCR (qPCR), a real-time PCR instrument (Light Cycler 480, Roche, Switzerland) was employed. The primer sets used in the gene expression study of *IL1β*, *IL10*, *IL8*, *TNFα*, *TLR5*, *iNOS*, *TGFβ*, *C3*, *NF-κB* and *MyD88* are mentioned in **Table 1**. For acting as a housekeeping gene, the *18S rRNA* was utilized to normalize the samples. Each sample contained 6 μL of nuclease-free water, 10 μL of 2× SYBR green I mix (A46110, Thermo Scientific, USA), 1 μL of forward primer (5 pmol), 1 μL of reverse primer (5 pmol) along with 2 μL of cDNA as a template for a total reaction volume of 20 μL. The thermal cycling program consisted of a 5 min pre-incubation at 95°C, followed by 40 cycles of amplification for 10 s at 95°C, 10 s of annealing at the gene-specific temperature (see Table 1) and 10 s of extension at 72 °C. At 95°C for 5 s, 65°C for 1 min and 97°C for 1 min, the specificity of qPCR was assessed using melt curve analysis. The samples were then allowed to cool for

Table 1. Sets of primers utilized in qPCR analysis to determine the mRNA expression of immune-related genes in fingerlings of koi carp.

Target gene	**Oligo sequence (5′–3′)**		**Annealing temperature**	**Product size**	**Source**
	Forward	**Reverse**			
IL1β	TTACAGTAAGACCAGCCTGA	AGGCTCGTCACTTAGTTTGT	60°C	89 bp	[29]
IL10	GCTGTCACGTCATGAACGAGAT	CCCGCTTGAGATCCTGAAATAT	60°C	132 bp	[30]
IL8	AGCCGACGCATTGGAAAACTCA	CATGGGGCTTTGTTGGCAATGA	60°C	175 bp	[31]
TNFα	GTGTCTACAGAAACCCTGGA	AGTAAATGCCGTCAGTAGGA	60°C	109 bp	[29]
TLR5	GAAGTAGTGAAAAGCACCTCGG	GATTTACATGCGTGGGCACT	60°C	103 bp	[32]
iNOS	AACAGGTCTGAAAGGGAATCCA	CATTATCTCTCATGTCCAGAGTCTCTTCT	60°C	101 bp	[30]
TGFβ	ACGCTTTATTCCCAACCAAA	GAAATCCTTGCTCTGCCTCA	60°C	97 bp	[30]
C3	GTCGGTCCTGGACTGTCTCT	AGTGCACTGCTTCTCCTGCT	60°C	113 bp	[29]
NF-κB	AACCAGGACCAGGCTTTCACT	CATGTAGCGGCCATAGGAATC	60°C	198 bp	[33]
MyD88	CGCCGAAATGATGGACTTCAC	TCTACTGTTGCCTCTGGACG	60°C	111 bp	[32]
18S rRNA	GAGTATGGTTGCAAAGCTGAAAC	AATCTGTCAATCCTTTCCGTGTCC	56°C	128 bp	[34]

10 s at 40°C. The results of the fold-change of expression with respect to the *18S rRNA* gene have been determined using the $2^{-\Delta\Delta CT}$ method.

2.12. Statistical analysis

To present all data, the means ± standard errors of the means (SEM) have been used. Before conducting statistical analysis, Kolmogorov-Smirnov's and Levene's tests were performed to assess the normal distribution as well as homogeneity of variance of the data, respectively. A one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test was performed using the SPSS statistical software (version 25.0) to evaluate the significance differences in each parameter. At $p < 0.05$, the significance levels were provided. Further, principal component analysis (PCA) was performed using Origin-Pro Graphing & Analysis software to evaluate the relationships among the expressed genes and their overall variation in liver and kidney tissues of fingerlings across the different experimental groups.

3. Results

3.1. Breeding performances

When DEHP concentrations got higher, the fertilization, hatching as well as survival of larvae rates decreased significantly ($p < 0.05$) contrasted with the control group (**Fig. 1**). In the control group, the rates of fertilization, hatching and larval survival were found to be 90 ± 2.62, 86 ± 2.36 and 88 ± 2.58 (%), respectively. However, these values were 64 ± 2.36, 62 ± 2.45 and 66 ± 2.62 (%), respectively in the higher DEHP treatment group (100 μg/L).

3.2. Growth parameters

The growth parameters, such as length gain, weight gain as well as specific growth rate were reduced significantly ($p < 0.05$) in the 40 days old fingerlings obtained from 100 and 10 μg/L of DEHP-treated groups (**Table 2**). However, compared to control group, the fingerlings of lower treated group (1 μg/L of DEHP) revealed no negative impact on growth performances. Moreover, during the rearing period of post larvae derived from various experimental groups, no mortality was recorded.

3.3. Accumulation of DEHP

DEHP accumulation was found in the testes of all males exposed to DEHP, but not in the control males (**Fig. 2**). The testes tissue of koi carp treated with 100 μg/L of DEHP (higher concentration) had significantly ($p < 0.05$) higher levels of DEHP, followed by 10 μg/L (medium concentration) and 1 μg/L (lower concentration) of DEHP-exposed groups. DEHP concentrations were measured with 12.46 ± 0.52 μg/g in the testes tissue of the males exposed to DEHP at 100 μg/L. In contrast, fingerlings of both the control and DEHP-exposed groups had below the detection limit of DEHP.

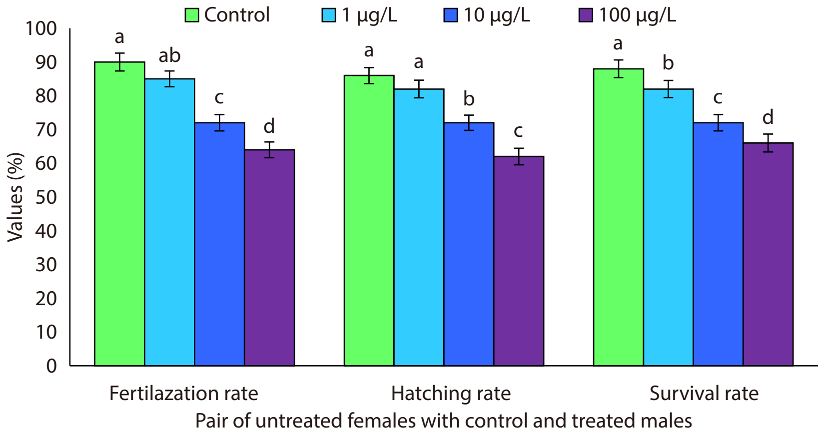


Fig. 1. The rates of fertilization, hatching and survival of larvae (%) of koi carp when untreated females were paired with control and nominal concentrations of DEHP-exposed (1, 10 and 100 μg/L for 150 days) males. Data are presented as mean ± SEM of three replicates (n = 6) and a significant difference at the 0.05 level is shown by values with unique superscript letters ('a' indicates the highest value, followed by 'b', 'c' and 'd').

Table 2. Growth parameters of fingerlings of koi carp obtained from pairing of untreated females with control and nominal concentrations of DEHP-exposed (1, 10 and 100 µg/L for 150 days) males. Data are presented as mean ± SEM of three replicates (n = 6) and a significant difference at the 0.05 level is shown by values with unique superscript letters ('a' indicates the highest value, followed by 'b', 'c' and 'd').

Parameters	Fingerlings of control group	Fingerlings of 1 µg/L group	Fingerlings of 10 µg/L group	Fingerlings of 100 µg/L group
Initial length (mm)	15.4 ± 1.15^{a}	15.2 ± 0.88^{a}	15.2 ± 1.02^{a}	15.0 ± 0.85^{a}
Final length (mm)	36.2 ± 1.82^{a}	35.4 ± 1.56^{a}	30.8 ± 1.44^{b}	30.2 ± 1.75^{b}
Length gain (mm)	20.8 ± 1.54^{a}	20.2 ± 1.45^{a}	15.6 ± 1.62^{b}	15.2 ± 1.42^{b}
Initial weight (mg)	35.4± 2.15^{a}	34.6 ± 2.22^{a}	34.4 ± 1.84^{a}	32.8 ± 1.75^{a}
Final weight (mg)	775.6 ± 34.48^{a}	726.8 ± 28.25^{ab}	658.6 ± 24.64^{c}	565.2 ± 26.5^{d}
Weight gain (mg)	740.2 ± 28.55^{a}	692.2 ± 25.68^{ab}	624.2 ± 26.22^{c}	532.4 ± 24.12^{d}
Specific growth rate (%/day)	10.28 ± 0.14^{a}	10.14 ± 0.12^{ab}	9.84 ± 012^{c}	9.48 ± 0.08^{d}
Mortality rate (%)	0	0	0	0

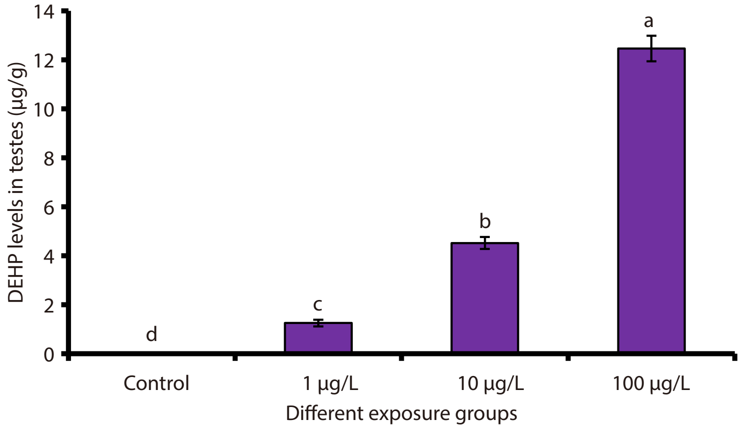


Fig. 2. DEHP accumulation (µg/g) in mature male koi carp testes after 150 days of exposure to nominal concentrations of 1, 10 and 100 µg/L of DEHP. Data are presented as mean ± SEM of three replicates (n = 6) and a significant difference at the 0.05 level is shown by values with unique superscript letters ('a' indicates the highest value, followed by 'b', 'c' and 'd').

3.4. Histological alternations

The liver tissue of fingerlings from the control group showed normal structural organization with hepatocytes exhibiting prominent nuclei and well-formed blood capillaries (**Fig. 3a**). In the paternally exposed fingerlings of 1 µg/L DEHP-treated group, liver tissue appeared largely normal, while only occa-

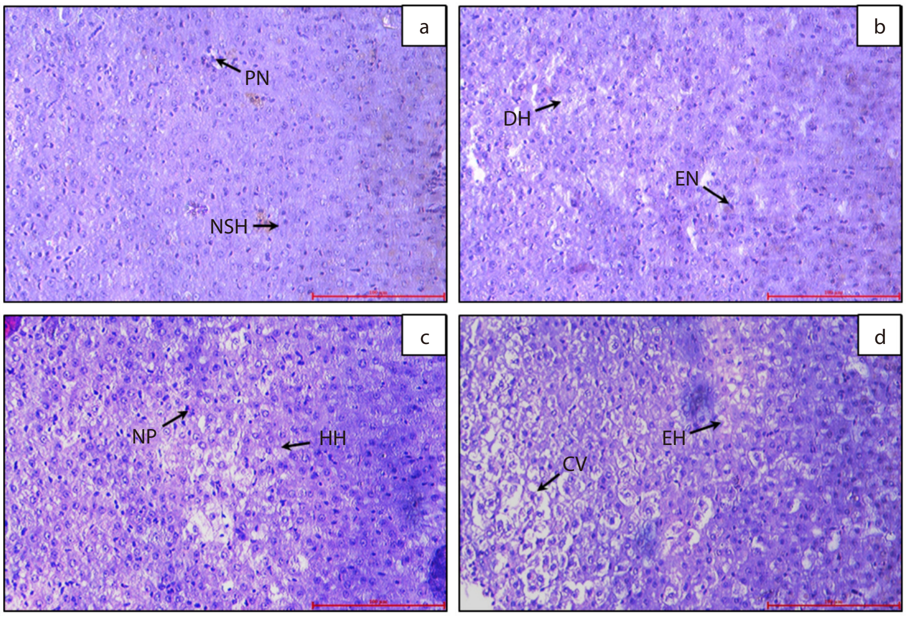


Fig. 3. H & E stained histological section of the liver in fingerlings of koi carp obtained from pairing of untreated females with control and nominal concentrations of DEHP-exposed (1, 10 and 100 µg/L for 150 days) males. (a) Fingerlings from control group shows the normal structure of hepatocyte (NSH) with prominent nucleus (PN). (b) Fingerlings from 1 µg/L of DEHP-treated group shows the degenerated hepatocyte (DH) with elongated nucleus (EN). (c) Fingerlings from 10 µg/L of DEHP-treated group shows the hemorrhagic hepatocytes (HH) and nuclear pyknosis (NP). (d) Fingerlings from 100 µg/L of DEHP-treated group shows the enucleated hepatocyte (EH) as well as cytoplasmic vacuolation (CV).

sional degenerated hepatocytes displaying elongated nuclei (**Fig. 3b**). Fingerlings from the 10 µg/L DEHP-exposed group exhibited hemorrhagic hepatocytes with nuclear pyknosis, indicating moderate cellular damage (**Fig. 3c**). The liver of fingerlings obtained from the group treated with 100 µg/L of DEHP displayed severe alterations, including enucleated hepatocytes with cytoplasmic vacuolation (**Fig. 3d**). Semi-quantitative scoring demonstrated a clear concentration-dependent increase in the severity of hepatic alterations in fingerlings from paternally treated groups (**Table 3**).

The kidney tissue of fingerlings from the control group showed normal cellular organization, featuring well-formed glomeruli encapsulated in Bowman's capsule and renal tubules with hematopoietic tissue dispersed in the interstitium (**Fig. 4a**). In the fingerlings obtained from the 1 µg/L DEHP-treated group, kidney tissue appeared largely normal, while only narrow tubular lumen (NTL) and mild renal tubule degeneration observed (**Fig. 4b**). The kidney of fingerlings derived from the group treated with 10 µg/L of DEHP displayed slight contraction of glomeruli and expansion of space inside Bowman's capsule along with moderate degeneration of renal tubules (**Fig. 4c**). Finally, marked edema around renal tubules, vacuolation in the glomerular tuft and shrunken glomeruli with dilated Bowman's capsule capillaries were evident in the fingerlings of the 100 µg/L DEHP-exposed group (**Fig. 4d**). Semi-quantitative scoring further confirmed a concentration-dependent increase in the severity of renal alterations across paternally exposed fingerlings (**Table 4**).

3.5. Immune and stress-response biomarker levels

The levels of HSP70 and IgM in fingerlings of the 10 and 100 µg/L (medium and higher concentrations, respectively) of DEHP-exposed groups were decreased significantly ($p < 0.05$). However, in fingerlings of the 1 µg/L of DEHP (lower concentration) treated group, the levels of HSP70 and IgM showed no significant difference ($p < 0.05$) with regard to the fingerlings of control group (**Fig. 5a and b**). In contrast, the HSP90 levels reduced significantly ($p < 0.05$) in fingerlings derived from pater-

Table 3. Semi-quantitative scoring of liver tissue alterations in fingerlings of koi carp obtained from pairing of untreated females with control and nominal concentrations of DEHP-exposed (1, 10 and 100 µg/L for 150 days) males.

Alterations in liver	Fingerlings of control group	Fingerlings of 1 µg/L group	Fingerlings of 10 µg/L group	Fingerlings of 100 µg/L group
Degenerated hepatocytes	–	+	++	+++
Nuclear elongation	–	+	++	++
Hemorrhagic hepatocytes	–	–	+	++
Nuclear pyknosis	–	–	+	++
Enucleated hepatocytes	–	–	–	+
Cytoplasmic vacuolation	–	–	–	++

Scoring: – = absent, + = mild, ++ = moderate and +++ = severe

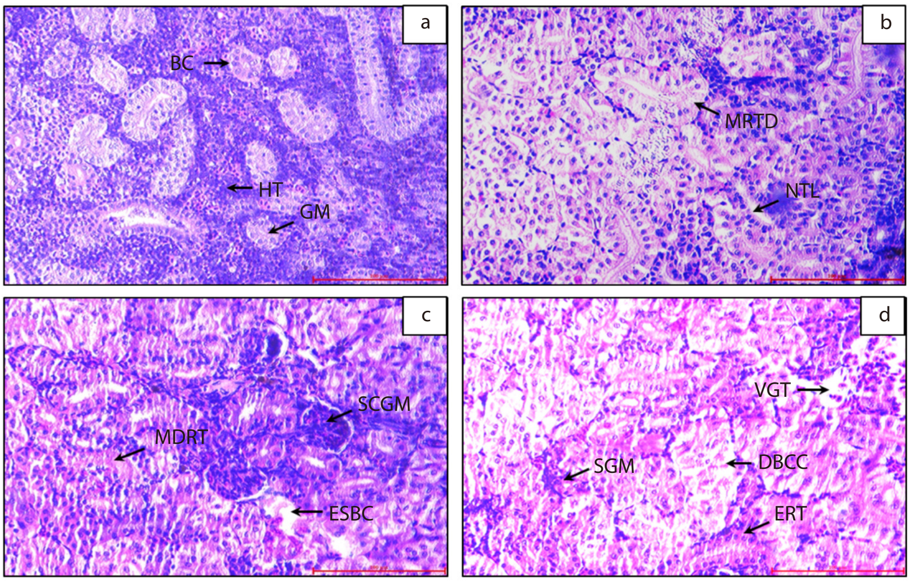


Fig. 4. H & E stained histological section of the kidney in fingerlings of koi carp obtained from pairing of untreated females with control and nominal concentrations of DEHP-exposed (1, 10 and 100 µg/L for 150 days) males. (a) Fingerlings from control group shows the normal structural organization of glomeruli (GM) encapsulated in Bowman's capsule (BC) and renal tubules with haematopoietic tissue (HT). (b) Fingerlings from 1 µg/L of DEHP-treated group shows the narrow tubular lumen (NTL) and mild renal tubule degeneration (MRTD). (c) Fingerlings from 10 µg/L of DEHP-treated group shows the slight contraction of glomeruli (SCGM), expansion of space inside Bowman's capsule (ESBC) along with moderate degeneration of renal tubules (MDRT). (d) Fingerlings from 100 µg/L of DEHP-treated group shows the marked edema around renal tubules (ERT), vacuolation in glomerular tuft (VGT) and shrunken glomerulus (SGM) with dilated Bowman's capsule capillaries (DBCC).

Table 4. Semi-quantitative scoring of kidney tissue alterations in fingerlings of koi carp obtained from pairing of untreated females with control and nominal concentrations of DEHP-exposed (1, 10 and 100 μg/L for 150 days) males.

Alterations in kidney	Fingerlings of control group	Fingerlings of 1 μg/L group	Fingerlings of 10 μg/L group	Fingerlings of 100 μg/L group
Narrow tubular lumen	–	+	+	++
Renal tubule degeneration	–	+	++	+++
Glomerular contraction/shrinkage	–	–	+	++
Bowman's space expansion	–	–	+	++
Edema around renal tubules	–	–	–	++
Vacuolation in glomerular tuft	–	–	+	++

Scoring: – = absent, + = mild, ++ = moderate and +++ = severe

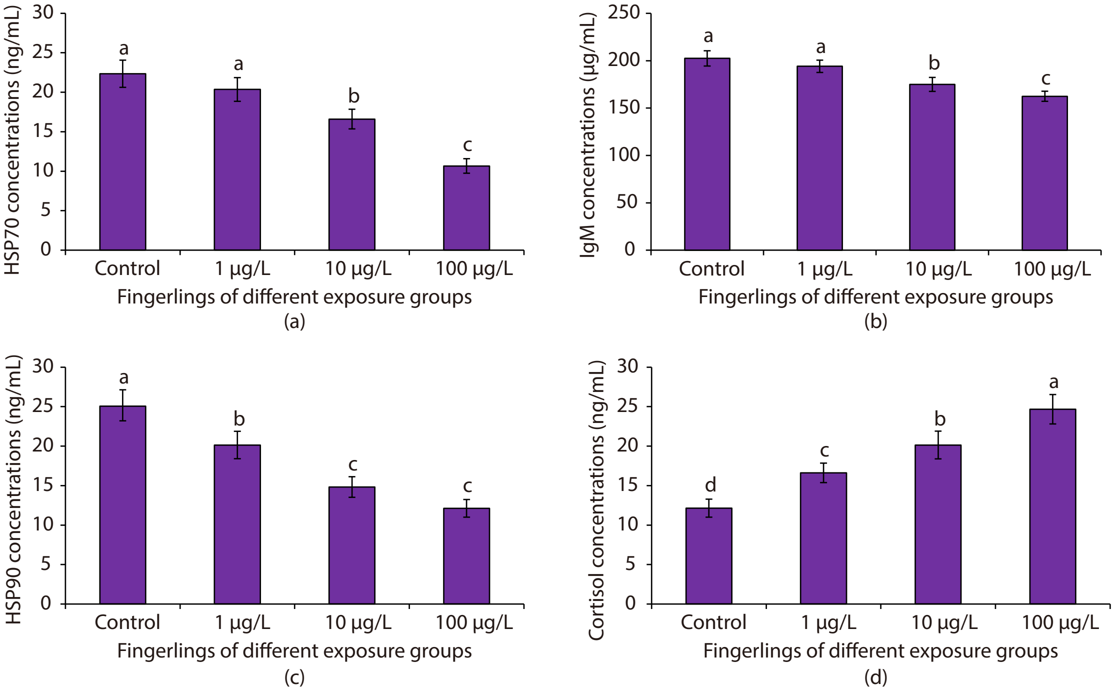


Fig. 5. The levels of HSP70 (ng/mL) (a), IgM (μg/mL) (b), HSP90 (ng/mL) (c) and cortisol (ng/mL) (d) in fingerlings of koi carp obtained from pairing of untreated females with control and nominal concentrations of DEHP-exposed (1, 10 and 100 μg/L for 150 days) males. Data are presented as mean ± SEM of three replicates (n = 6) and a significant difference at the 0.05 level is shown by values with unique superscript letters ('a' indicates the highest value, followed by 'b', 'c' and 'd').

nally exposed groups, as the concentrations of DEHP raised (**Fig. 5c**). On the contrary, it was observed that fingerlings obtained from exposed groups with each concentration of DEHP exhibited significantly ($p < 0.05$) higher levels of cortisol with respect to the fingerlings of control group (**Fig. 5d**).

3.6. mRNA expression of immune genes

Comparing the fingerlings of DEHP-treated groups to the control group, the hepatic and renal expression of immune-related genes showed clear alterations. mRNA analysis revealed that most of the genes examined, including *IL1β*, *IL8*, *TNFα*, *TLR5*, *iNOS*, *C3*, *NF-κB* and *MyD88* were significantly upregulated ($p < 0.05$) in both the liver and kidney of fingerlings from the medium (10 μg/L) and higher concentration (100 μg/L) exposure groups (**Fig. 6a**-**h**). Notably, *IL1β*, *TLR5* and *NF-κB* also exhibited significant upregulation ($p < 0.05$) in both tissues of fingerlings obtained from the lower concentration (1 μg/L) paternal exposure group. In contrast, *IL10* and *TGFβ* mRNA levels were significantly reduced ($p < 0.05$) in the liver and kidney of fingerlings derived from the 10 and 100 μg/L DEHP-exposed groups (**Fig. 7a and b**). A reduction in *TGFβ* expression was also observed in the liver, but not in the kidney of fingerlings obtained from the 1 μg/L paternal exposure group.

Principal component analysis (PCA) revealed that the first component contributed 95.64% to hepatic immune-related gene expression among fingerlings from different paternal exposure groups, while the second component explained 3.40% of the total variance (**Fig. 8a**). Similarly, in kidney tissue of fingerlings from various exposure groups, the first and second components accounted for 94.22% and 4.18% of the overall variance in gene expression, respectively (**Fig. 8b**). In the biplots, genes that projected in the same direction as a principal component showed positive contributions, indicating coordinated expression patterns. In contrast, genes oriented in the opposite direction were considered to have negative contributions, reflecting opposing variations relative to that component. In both the liver and kidney, *IL10* and *TGFβ* from fingerlings in the control and 1 μg/L DEHP-exposed groups showed positive contributions, which was consistent with a relatively

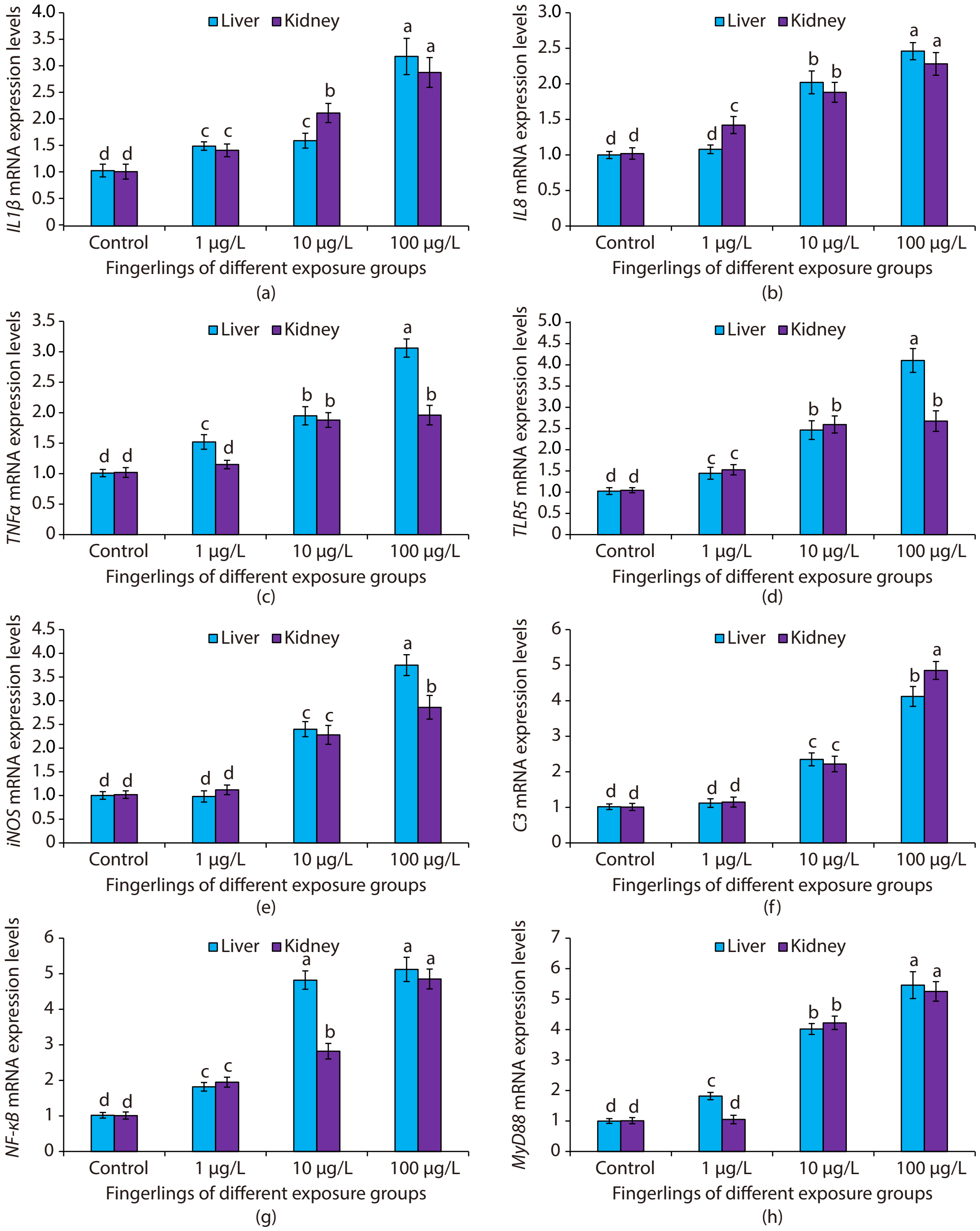
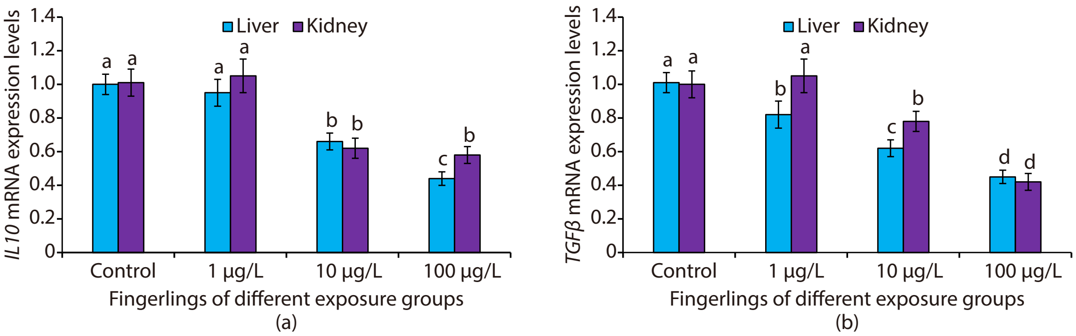


Fig. 6. Upregulated mRNA expression of *IL1β* (a), *IL8* (b), *TNFα* (c), *TLR5* (d), *iNOS* (e), *C3* (f), *NF-κB* (g) and *MyD88* (h) in liver and kidney tissue of fingerlings of koi carp obtained from pairing of untreated females with control and nominal concentrations of DEHP-exposed (1, 10 and 100 μg/L for 150 days) males. Data are presented as mean ± SEM of three replicates (n = 6) and a significant difference at the 0.05 level is shown by values with unique superscript letters ('a' indicates the highest value, followed by 'b', 'c' and 'd').

balanced anti-inflammatory immune profile. However, these genes (*IL10 and TGFβ*) shifted to negative contributions in both tissues of fingerlings from the 10 μg/L and 100 μg/L exposure groups, indicating a suppression of anti-inflammatory signaling. Additionally, *IL1β* in the liver and *C3* in the kidney of fingerlings from the 10 μg/L DEHP-treated group contributed negatively, which aligned with increased pro-inflammatory or complement activity. Overall, the PCA showed a clear concentration-dependent shift from a coordinated anti-inflammatory gene expression pattern in the control and lower concentration groups to a more pro-inflammatory and unregulated immune profile in paternally exposed offspring.

4. Discussion

Growing evidence suggests that exposure of aquatic organisms to endocrine-disrupting chemicals (EDCs), such as DEHP can lead to a range of adverse health effects [35-39]. Importantly, the absence of overt toxicity in directly exposed individuals does not preclude negative outcomes in their unexposed offspring, which highlights a major challenge for environmental risk assessment [23]. In the present study, we examined the effects of prolonged DEHP exposure (150 days) at environmentally relevant concentrations (1, 10 and 100 μg/L) on the breeding performance of koi carp as well as on immune-related



(a) (b)

Fig. 7. Downregulated mRNA expression of *IL10* (a) and *TGFβ* (b) in liver and kidney tissue of fingerlings of koi carp obtained from pairing of untreated females with control and nominal concentrations of DEHP-exposed (1, 10 and 100 µg/L for 150 days) males. Data are presented as mean ± SEM of three replicates (n = 6) and a significant difference at the 0.05 level is shown by values with unique superscript letters ('a' indicates the highest value, followed by 'b', 'c' and 'd').

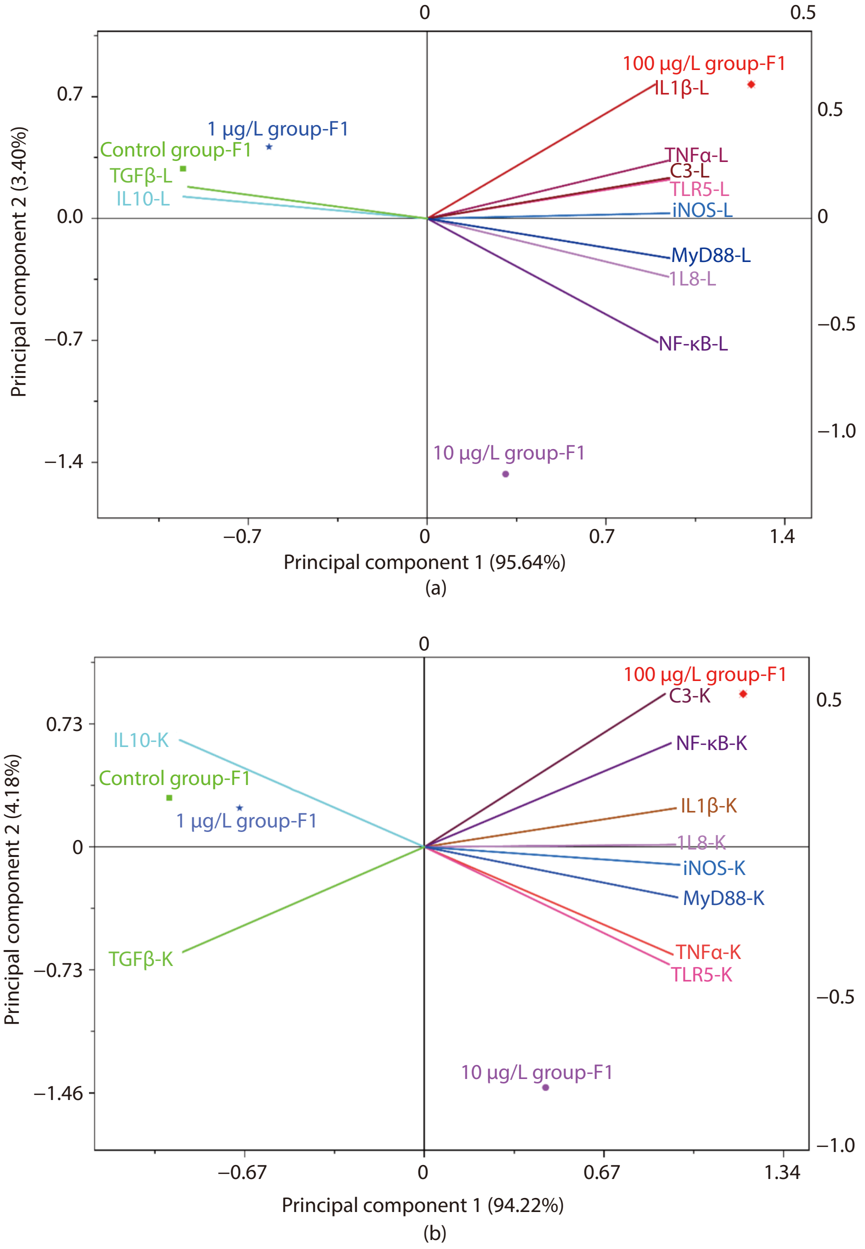


Fig. 8. Biplot on expression patterns of different genes through PCA studied in liver (a) and kidney (b) tissue of fingerlings of koi carp obtained from pairing of untreated females with control and nominal concentrations of DEHP-exposed (1, 10 and 100 µg/L for 150 days) males.

parameters in their offspring. The extended exposure period encompassed gonadal development and the active reproductive phase of male koi carp, a seasonally breeding species, thereby providing a realistic model of chronic environmental exposure. Compared with short-term exposure studies (20–60 days), this long-term design more accurately reflects continuous contamination in natural aquatic systems and allows evaluation of cumulative effects on male reproductive physiology and paternal-mediated transgenerational outcomes. By covering the full reproductive cycle, the present work demonstrates how paternal DEHP exposure can influence not only reproductive success, but also offspring health, including growth performance, immune competence and tissue integrity.

In the current investigation, when exposed males were paired with untreated females, there were statistically significant reductions in fertilization, hatching and larval survival rates, indicating that paternal exposure to DEHP significantly impaired reproductive performance. Similar outcomes have been reported in *Danio rerio*, where paternal DEHP treatment led to decreased fertilization and hatching success following exposure to 0.2 and 20 µg/L for 21 days [40] as well as 0.5 µg/L for 180 days [41]. The decline in male breeding performance observed here is consistent with DEHP-induced disruption of androgenic signaling, including reduced testicular expression of the androgen receptor (*Ar*) and lower levels of 11-ketotestosterone, a central regulator of the hypothalamic-pituitary-gonadal (H-P-G) axis [15, 18]. Reduced spermatogenesis and impaired fertility have been directly associated with suppression of androgen signaling [42]. Furthermore, the GC-MS technique was used to evaluate the accumulation of DEHP in the testes of exposed males and possible transfer to their progeny. Across all concentrations, DEHP was readily found in the testes of exposed males, but remained below detectable levels in their offspring. In our earlier study [18], DEHP exposure for 60 days at 1–100 µg/L accumulated remarkably in the testes of *C. carpio* in a concentration-dependent manner. However, none of the studies found the presence of any phthalate compound in the progeny of exposed fish. The transgenerational effects seen in this study are most likely mediated through epigenetic changes in the paternal germline rather than by direct chemical transfer, even though there was no detectable DEHP in the offspring tissues.

Recent findings indicate that paternal exposure to EDCs, including phthalates can induce persistent epigenetic modifications in sperm, such as alterations in DNA methylation, histone modifications and non-coding RNA profiles, thereby influencing embryonic development, immune function and stress responses [43-45]. In teleost fishes, such epigenetic changes have been linked to impaired offspring growth, reduced immune competence and diminished stress resilience [46, 47]. Similar paternal inheritance mechanisms have also been reported in mammals, where phthalate-induced alterations in sperm DNA methylation and small RNA profiles are associated with immune and metabolic disturbances in the progeny [48, 49]. Consistent with this framework, larvae derived from DEHP-exposed males were reared under controlled conditions to assess the developmental consequences of paternal exposure. Growth performance, including length gain, weight gain and specific growth rates were significantly reduced in 40 days old fingerlings in a concentration-dependent manner, indicating that parental DEHP exposure adversely affects offspring growth trajectories. Previous studies have documented growth inhibition following direct DEHP exposure in early life stages, such as reduced larval growth in *Poecilia reticulata* after 14 days at 10 µg/L [50] or decreased body length and weight in *Oryzias latipes* larvae following 21 days of exposure to 20–200 µg/L [51]. The present findings demonstrate that similar developmental impairments can occur even in the absence of direct chemical exposure. Histopathological analysis further revealed pronounced alterations in the liver and kidney tissues of fingerlings derived from DEHP-exposed males. Liver plays a primary role in the metabolism and excretion of xenobiotic compounds with morphological alternations [52, 53]. Whereas, kidney is an essential organ involved in the metabolism and elimination of exogenous toxic substances [54, 55]. The observed changes in cellular architecture in these organs reflect the impact of paternal DEHP exposure. Comparable hepatic alterations, including cytoplasmic degeneration, nuclear deformation, vacuolization and necrosis have been reported in *C. carpio* following direct DEHP exposure at 10–1000 µg/L for 30 days [14]. Severe renal lesions, such as tubular degeneration, glomerular condensation and pyknotic nuclei have also been documented in *Clarias gariepinus* after 30 days of exposure to 40–80 µg/L DEP [56]. Moreover, these findings indicate that chronic paternal DEHP exposure can induce persistent histopathological alterations in key metabolic and excretory organs of offspring. When considered alongside impaired reproductive performance, reduced growth and immune dysregulation, the results strongly suggest that the adverse outcomes observed in the progeny are mediated by heritable epigenetic modifications resulting from chronic paternal exposure to DEHP.

The established methodology for assessing the acute or chronic toxicity of synthetic chemicals in aquatic animals often relies on the measurement of stress biomarkers, including HSP70, HSP90 and cortisol as well as immunological responses via IgM [57, 58]. Heat shock proteins (HSPs) play a central role in intracellular stress responses and the maintenance of protein homeostasis. Among HSPs, HSP70 and HSP90 are particularly important for protecting cells against oxidative damage [59]. In the present study, fingerlings derived from DEHP-exposed groups exhibited greatest reductions in HSP70 and HSP90 levels, indicating that the protective mechanisms provided by these proteins were compromised. IgM, a key immunoglobulin serves as an important indicator of immune competence and contributes to specific defense mechanisms [60]. Our results further demonstrated that IgM levels in paternally exposed fingerlings decreased significantly with increasing DEHP concentrations, suggesting suppression of innate immune responses. Cortisol, a primary corticosteroid released in response to stress is typically elevated under conditions perceived as threatening. The status of fish health can be assessed by measuring cortisol levels because prolonged elevated levels are negatively linked to growth, development and immunity [61]. In this study, cortisol levels were elevated in fingerlings from DEHP-exposed groups, indicating a heightened stress response and potential physiological disturbances during development.

Gene expression changes are considered as primary and important response in toxicological investigations, making genomic analysis a valuable tool [62, 63]. As a result, toxicant effects at environmentally relevant concentrations would be detected and measured early [64, 65]. High-sensitivity, mechanism-based markers that can potentially indicate long-term adverse effects are expected to be observed using gene expression techniques [66, 67]. Proper expression of inflammatory cytokines (both pro and anti) is essential for maintaining immune system homeostasis and responding to inflammation [68, 69]. Chronic inflammation has been linked to alterations in cytokine profiles, which may ultimately result in autoimmune sickness [70, 71]. Pro-inflammatory cytokines, including *IL1β*, *IL8* and *TNFα* promote inflammation, while anti-inflammatory cytokines, such as *IL10* and *TGFβ* dampen the response and promote tissue repair [72, 73]. Others immune genes includes *TLR5*, *iNOS*, *C3*, *NF-κB* and *MyD88* plays crucial role in activating immune response and initiating inflammatory responses [74, 75]. In the present investigation, paternal exposure of DEHP modulated the activation of immune response in offspring leading to upregulated expression of pro-inflammatory genes (*IL1β*, *IL8*, *TNFα*, *TLR5*, *iNOS*, *C3*, *NF-κB* and *MyD88*) along with downregulated expression of anti-inflammatory genes (*IL10* and *TGFβ*). After direct exposure to DEHP, the alternations in immune-related gene expressions were reported in *Oryzias melastigma* [76] and *D. rerio* [77]. Further, exposure to DEHP remarkably accelerated the activation of TLR4, MyD88 and NF-κB pathways in *Ctenopharyngodon idella* [78]. The mRNA expression data from the current study indicate a pronounced shift toward pro-inflammatory dominance in fingerlings derived from DEHP-exposed males, which is consistent with the magnitude of histopathological alterations and biomarker responses observed in the offspring. Specifically, the upregulation of pro-inflammatory cytokines, such as *IL1β* and *TNFα* may underlie the hepatic and renal tissue damage, while the downregulation of anti-inflammatory mediators, i.e., *IL10* and *TGFβ* are consistent with impaired resolution of inflammation. In parallel, the sustained elevation of cortisol observed in these fingerlings may reflect chronic physiological stress, further exacerbating immune dysregulation. Collectively, these molecular and biochemical responses provide a mechanistic framework linking paternal DEHP exposure to immune imbalance, tissue pathology and altered stress responses in the offspring.

Overall, integrated analyses of different biological endpoints performed in this study clearly show a concentration-dependent impact associated with paternal DEHP exposure. As the concentrations of DEHP rose, the adverse impacts on most parameters, such as breeding performance, DEHP accumulation, offspring growth rates, biomarker responses and immune-related gene expressions were progressively intensified. Disruptions that were most consistent were observed among the groups of 10 and 100 μg/L exposures, suggesting a kind of threshold rather than a strictly linear concentration-response relationship, whereas the 1 μg/L group mostly showed effects that were either at an intermediate level or non-significant. This is especially important for environmental risk assessment, since the pattern indicates that low levels of DEHP exposure might cause very few biological effects, while higher levels could lead to substantial physiological, developmental and immunological changes. All these findings highlight the potential ecological and intergenerational risks associated with prolonged DEHP contamination in aquatic ecosystems.

5. Conclusion

The present study demonstrates that environmentally relevant exposure of DEHP to mature male koi carp (*C. carpio*) impairs the breeding performance and also reduces the immune health of the F1 generation. Following 150 days of chronic exposure to DEHP found to be capable of reducing the rates of fertilization, hatching as well as larval survival. The findings of this research reveal that paternal exposure to DEHP influences the growth parameters, levels of immune and stress-response biomarkers and also the mRNA expression of numerous immune-related genes in their offspring. It has been shown that fingerlings from the higher DEHP exposure (100 μg/L) group exhibit the strongest effects. Overall, the results of this investigation are helpful and proficient biomarkers that might be utilized for screening the detrimental impacts of any synthetic chemical. Moreover, the outcomes of current study indicate that the effects of DEHP are not limited in exposed animals, but also extend to their offspring. These findings emphasize the necessity for long-term, transgenerational risk evaluations in ecotoxicology and aquaculture management as well as regulatory attention to paternal toxicant exposure. Finally, this research could be crucial for upcoming molecular research on the offspring of other animals after parental exposure.

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Conflicts of Interest

The authors declare that there are no conflicts of interest in this work.

Data Availability

All data needed to support the conclusions in the paper are presented in the manuscript and/or the Supplementary Materials. Additional data related to this paper may be requested from the corresponding author upon request.

Ethical Approval

Ethical considerations and animal handling protocols followed in this study have been described in the Materials and Methods section.

Electronic Supplementary Material (ESM)

The online version contains supplementary material available at https://doi.org/10.26599/ECS.2026.9600010

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