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Data Article

Experimental data-sets on sex reversal and histopathological assessment of potential endocrine disrupting effects of graphene oxide on Japanese medaka (*Oryzias latipes*) larvae at the onset of maturity

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ABSTRACT

The datasets of this article present the experimental parameters resulting from the assessment of sex reversal (SR) as a biomarker of endocrine disrupting effect of graphene oxide (GO), together with the histopathological assessment of ovary, testis, liver and kidneys of medaka larvae. These data sets support the published article "Sex-reversal and histopathological assessment of potential endocrine-disrupting effect of graphene oxide on Japanese medaka (*Oryzias larvae*) larvae." The experiments were conducted on one day-post hatch (dph) Japanese medaka fries (orange-red variety) exposed to different concentrations of GO (2.5–20 mg/L) by immersion in embryo-rearing medium (ERM) for 96 h under laboratory conditions (25 ± 1 °C; light cycle 16 h light: 8 h dark). No food was given during the GO-exposure period. Controls (no GO) were identically maintained in ERM. After treatment, the larvae were maintained in balanced salt solution (BSS) with feeding and allowed to grow for 6 more weeks in a GO-free environment. On 47 dph, the larvae were anesthetized in MS-222, and the total length (mm) and body

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weight (mg) were recorded. For histopathological and phenotypic sex assessments, after sacrifice, the body excluding post-anal tail was preserved in 4% paraformaldehyde containing 0.05% Tween 20; ovary, testis, liver and kidneys were evaluated in 5 µm thick sections stained on haematoxylin eosin (HE) following OECD guidelines. The photomicrographs of sections were made using either an Olympus B-max 40 microscope attached to a camera with Q-capture Pro 7 software or an Olympus CKX53 inverted microscope with DP22 camera and CellSens software. A minimum 3 images of gonads in different regions were further analysed by imagej software and used for counting spermatogonia (SPG) and spermatocytes (SPT) in testis as well as perinucleolar (PNO) and cortical alveolar (CAO) oocytes in ovary. Data were expressed as number of SPG or SPT/mm^2 testis and % CAO or PNO in an ovary. Preserved tail in TRI reagent was used for genomic DNA extraction and the genetic sex was assessed by genotyping Y chromosome-specific male sex-determining gene *dmy*. Two different sets of buffers and primers were used and the reactions were conducted in a thermal cycler. The amplified products were separated in 2% agarose gel containing 0.01% ethidium bromide. The gels were viewed on an UV illuminator and the genotypes were identified by visual inspection. The first primer set amplified a 355 bp product for XY genotypes and no amplification for XX. The second set of primers amplified two products; one at 1249 bp and another at 986 bp for XY, and one product at 1249 bp for XX. Experimental data were expressed as means ± SD or SEM, analysed either by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's multiple comparison test or unpaired parametric '*t*' test including Welch's correction, if distributed normally (lengths and weights), or by Kruskal-Wallis test followed by Man-Whitney's test as post hoc test, if data (stromal follicles in ovary and SPGs and SPTs in testis) did not meet the criteria of using a parametric test. Statistically significant difference were considered for $p \leq 0.05$.

Specifications Table

Subject	Pharmacology, Toxicology and Pharmaceutical Sciences
Specific subject area	Toxicology
Type of data	Table Image Graph Figure
How data were acquired	Ultra sonicator, LPX 750 (Cole Parmer) JEM-2100F Transmission electron microscope (TEM) (JOEL 2010, USA) Microtome (Olympus cut 4055) Olympus B-max 40 microscope attached to a camera with Q-capture Pro-7 software (Media Cybernetics) Olympus CKX53 inverted microscope with DP22 camera and CellSens software (Hunt optics & Imaging, Pittsburgh, PA, USA)

(*continued on next page*)

	Thermal cycler (Technie, Cole Parmer, Chicago, IL, USA) UV illuminator and vertical gel electrophoresis apparatus (Thermo Fisher Scientific, St. Louis, MO) Smartphone digital camera GraphPad Prism 7 (San Diego, CA, USA) imagej (imagej.nih.gov)
Data format	Raw Analyzed
Parameters for data collection	GO obtained commercially, sonicated in ERM, characterized by TEM. Larval growth after GO exposure (96h without food) and depuration in a GO-free environment (6 weeks with food), was determined by linear length and weight. Phenotypic sex and effects on testis, ovary, liver, and kidney were evaluated histologically. Distribution of stromal follicles in ovary, and SPG and SPT in testis were analyzed by imageJ software. Genotypic sex was determined by genotyping *dmy* [1].
Description of data collection	GO obtained from Sigma (St. louis MO), sonicated in ERM, characterized by TEM. One-dph Japanese medaka fries were immersed in different concentration of GO (2.5–20 mg/L) dispersed in ERM for 96 h without food and depurated in BSS in a GO-free environment under laboratory conditions for 6 weeks with food. On 47 dph, lengths and weights were measured; the phenotypic sex and the effects on testis, ovary, liver, and kidney were evaluated histologically in 5 µm thick HE stained sections. Distribution of stromal follicles in ovary, and SPG and SPT in testis were analyzed by imagej software. Genotypic sex was determined by genotyping dmy gene. Statistical analysis was done by using GrapPad Prism software [1].
Data source location	RCMI Centre for Environmental Health, Jackson State University, Jackson, MS 39217, USA.
Data accessibility	With the article
Related research article	Author' s names: Anitha Myla, Asok K Dasmahapatra, Pau B. Tchounwou [1] Title: Sex-reversal and histopathological assessment of potential endocrine-disrupting effects of graphene oxide in Japanese medaka (Oryzias latipes) larvae Journal: Chemosphere 279(2021) 130768; https://doi.org/10.1016/j.chemosphere.2021.130768

Value of the Data

- Sex reversal (SR) is a significant end point in the evaluation of EDCs disposed in the aquatic ecosystem. Our data on SR clearly indicate that in Japanese medaka (*Oryzias latipes*), an environmentally relevant model used in EDC studies, only XX genotypes have the potential to reverse sex (phenotypic testis instead of ovary) and GO was ineffective in inducing SR in Japanese medaka and no apparent EDC effects on testis, ovary, liver and kidneys during gonadal maturation.
- The data will be useful to the U.S. Government agencies such as Environmental Protection agency (EPA), Food and Drug administration (FDA), National Institutes of Health (NIH), Public Health Departments in different States of the USA and many investigators including environmental biologists, nanotoxicologists, drug-designers, cancer researchers, endocrinologists, fish biologists, and biomedical scientists involved in drug development and nanomedicine.
- The data generated from this research will be useful for determining the LC_{50}/LD_{50} of GO in animals living in any aquatic ecosystem, as well as the no observed effect level (NOEL), or the lowest observed effect concentration (LOEC) in laboratory experiments. Moreover, the data generated in this study indicate that SR is specific to the genotypes of an animal. Although our study indicates SR only in XX genotypes, it does not preclude to use XY genotypes during EDC studies.

- The experimental approach we used in the present experiment will be more convenient to the Organization of Economic Cooperation and Development (OECD) or other agencies such as US EPA to set guidelines for regulating the disposal of GO or GO-containing compounds in the environment, as well as to determine the toxic potential of not only GO or related nanocarbons but also of other toxic chemicals such as pesticides/herbicides on fish or other aquatic organisms.
- The cutting-edge data on larval growth, the genotyping technique we developed, the information on growth, SR and distribution of SPG/SPT in testis or PNOs/CAOs in the ovary generated through this research on medaka fish larvae are unique and can be shared within the scientific communities especially among toxicologists, reproductive/developmental biologists which is more convenient during the design of experiments and analysis of research results.

1. Data Description

The GO obtained from commercial source (2 mg/ml; dispersed in water) was diluted to 20 mg/L (100 times) by ERM and sonicated for 5 min (2s on-1s off pulse, 225 w) in ice temperature by a probe sonicator (ultrasonicator LPX 750, Cole Parmer, Chicago, IL, USA) and further checked under TEM at the facilities available at the Jackson State University (JSU), Jackson, MS, USA. The sonicated GO was further diluted to desired concentrations (10 mg/L, 5 mg/L and 2.5 mg/L) using ERM, and applied directly (1 ml/ fries) to 1 dph fries kept in 13 × 75 mm glass tubes and maintained in a waterbath at 25 ± 1 °C with 16 h light and 8 h dark light cycle. No food was given to the fries during GO exposure period (96h continuously). The controls were maintained in ERM only. The media (with and without GO) was refreshed everyday.

On 47th dph the larvae were anesthetized in MS222, weight to the nearest mg and the total lengths (mm) were measured using a ruler (Fig. 2, Table 1a and b, and supplementary Table (ST)-1). The larvae were separated into different groups depending on the phenotype (based on gonad histology) and genotypes (based on *dmy* genotyping) of the larvae. The phenotypic sex of the larvae (testis or ovary) on 47th dph was determined by following standard histological techniques (Figs. 3–4) as described previously in our laboratory [1–3] and the genotypic sex was determined by genotyping tail DNA [4] and visualyzed on an UV illuminator after electrophoretically separated the amplified products (Fig. 4h)

The total lengths of the larvae were measured in mm (Table 1A). The body weight of the larva were expressed as mg (Table 1b). The data are expressed as mean± SD. (n) = number of observations. One-day post hatch larvae were exposed to different concentration of GO (2.5–20 mg/L) in ERM continuously for 96h. No food was provided to the larvae during exposure period. After treatment, the larvae were maintained in BSS with regular feeding until 47 dpf in a GO-free environment. Before sacrifice, the larvae were anesthetized in MS 222; the linear

Table 1a
Effect of GO exposure on the total length of the larvae at 47th dph:

	Control	2.5 mg/L	5.0 mg/L	10. 0 mg/L	20.0 mg/L
XY genotype	20.50 ± 1.92 (n = 4)	19.2 ± 1.30 (n = 5)	20.33 ± 1.53 (n = 3)	19.25 ± 2.06 (n = 4)	20.20 ± 1.79 (n = 5)
XX genotype	19.00 ± 3.37 (n = 4)	18.00 ± 2.00 (n = 3)	20.00 ± 2.94 (n = 4)	19.33 ± 1.16 (n = 3)	20.00 ± 0.00 (n = 3)
phenotypic testis	19.17 ± 2.71 (n = 6)	19.00 ± 1.27 (n = 6)	20.00 ± 1.41 (n = 6)	19.17 ± 1.72 (n = 6)	20.17 ± 1.60 (n = 6)
phenotypic ovary	21.50 ± 2.12 (n = 2)	18.00 ± 2.83 (n = 2)	19.5 ± 4.95 (n = 2)	20.00 ± 0.00 (n = 1)	20.00 ± 0.00 (n = 2)
Sex reversed testis	16.5 ± 2.12 (n = 2)	18.00 ± 0.00 (n = 1)	20.5 ± 0.71 (n = 2)	19.00 ± 1.41 (n = 2)	20.00 ± 0.00 (n = 1)

Table 1b
Effects of GO exposure on the body weight of the larvae at 47th dph.

	Control	2.5 mg/L	5.0 mg/L	10.0 mg/L	20 mg/L
XY genotype	79.75 ± 17.85 ($n = 4$)	70.20 ± 17.341 ($n = 5$)	80.67 ± 11.72 ($n = 5$)	68.50 ± 22.69 ($n = 4$)	78.40 ± 27.38 ($n = 5$)
XX genotype	65.25 ± 29.09 ($n = 4$)	65.67 ± 17.16 ($n = 3$)	85.60 ± 24.81 ($n = 5$)	80.00 ± 17.00 ($n = 3$)	80.00 ± 7.55 ($n = 3$)
Testis	67.50 ± 24.88 ($n = 6$)	69.00 ± 15.79 ($n = 6$)	85.2 ± 11.19 ($n = 5$)	72.33 ± 21.45 ($n = 6$)	78.83 ± 24.51 ($n = 6$)
Ovary	87.50 ± 14.85 ($n = 2$)	67.00 ± 24.04 ($n = 2$)	92.00 ± 8.49 ($n = 2$)	80.00 ± 0.00 ($n = 1$)	81.00 ± 0.00 ($n = 1$)
Sex-reversed testis	43.00 ± 18.38 ($n = 2$)	63.00 ± 0.00 ($n = 1$)	80.00 ± 24.04 ($n = 2$)	80.00 ± 24.04 ($n = 2$)	79.50 ± 10.61 ($n = 2$)

Table 2
Effects of GO exposure on the distribution of SPGs and SPTs in the phenotypic testis of XY and XX genotypes.

Genotype	Testicular components	Control	GO concentrations (mg/L) 2.5	5.0	10.0	20.0
XY	SPG	249.6 ± 10.07 ($n = 66$)	296.1 ± 12.79 ($n = 102$)	269.0 ± 10.75 ($n = 80$)	331.7 ± 13.12 ($n = 75$)$^{*\#}$	226.9 ± 7.86 ($n = 98$)$^{\#\&}$
	SPT	430.8 ± 8.93 ($n = 85$)	441.6 ± 9.69 ($n = 109$)	406.9 ± 6.32 ($n = 124$)$^{*\#}$	455.5 ± 9.54 ($n = 94$)$^{\$}$	413.9 ± 7.65 ($n = 137$)$^{\#\&}$
XX	SPG	270.5 ± 8.27 ($n = 75$)	256.0 ± 10.76 ($n = 48$)	252.3 ± 11.17 ($n = 43$)	268.8 ± 18.28 ($n = 33$)	225.2 ± 8.90 ($n = 51$)$^{*\#\&}$
	SPT	364.6 ± 14.76 ($n = 34$)	396.2 ± 14.48 ($n = 30$)	444.4 ± 12.76 ($n = 61$)$^{*\#}$	393.1 ± 18.12 ($n = 35$)$^{\$}$	403.2 ± 14.38 ($n = 33$)$^{*\$}$

The SPGs and SPTs data were expressed as mean ± SEM of the number of cells $X10^4/mm^2$ testis. The symbols used in superscripts after the data indicate significant difference; asterisks (*) indicates that the data are significantly different from the control group, pound (#) indicates the data are significantly different from the 2.5 mg/L group, dollar ($) indicates the data are significantly different from 5.0 mg/L group, and the symbol (&) indicate that the data are significantly different from 10.0 mg/L group.

total length (Table 1a) of each larva was measured using a ruler and the weights (Table 1b) were recorded. The phenotypic sex of the larvae was determined by histology and the genetic sex was assessed by genotyping dmy gene (Figs. 3, 4).

The genotypic sex of the medaka larvae after 47 dph was determined by extracting tail DNA by HotSHORT technique [4] and genotyping the extracted DNA targeting male sex determining gene *dmy* [1] by a PCR-based method. The amplified product was viwed in 2% agarose gel electrophoresis containg 0.01% ethidium bromide and viewed on an UV illuminator.

2. Experimental Design, Materials and Methods

All the experimental animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Jackson State University, Jackson, MS.

2.1. Animal maintenance and exposure to GO

The animal maintenance and details of the medaka colony maintained at the Medaka fish culture facility at the Jackson State University were described previously [1–3]. Briefly, the adult

Japanese medaka fish (Orange red varieties, Hd-rR strain) fish colony we developed and maintained at the Jackson State University, Jackson, MS, USA, were obtained from the University of Mississippi, University, Mississippi, USA, by a protocol transfer agreement. The adult fish used as breeders (females and males) were maintained in 35 L glass tanks in 25 L balanced salt solution (BSS; 17 mM NaCl, 0.4 mM KCl, 0.3 mM $MgSO_4$, 0.3 mM $CaCl_2$, pH 7.4) at 25 ± 1 °C with 16 h L: 8h D light cycle. The media was recirculated through pumps and filtered through disposable bio and carbon filters. BSS was replaced on every two-three weeks, if the pH is above 7.5 and the ammonia concentration is above 3. The fish breed successfully in this environment and the eggs were generally collected within 1–3 h after the light was turned on. The collected embryos were gently separated from the clutch and separate them as individual egg after rubbing on a metal sieve. The viable, fertilized eggs were separated from the dead and damaged embryos under a binocular scope. The viable embryos were reared in embryo-rearing medium (ERM; 17 mM NaCl, 0.4 mM KCl, 0.6 mM $MgSO_4$, 0.36 mM $CaCl_2$, pH 7.4). The cultured embryos generally hatched within 10 days after post fertilization (dph). The development and staging of the experimental embryos and larvae were conducted as previously described [5]. Any embryos hatched after 14 day post fertilization (dpf) was excluded from the GO exposure.

Medaka fries (Stage 40, 1st fry stage) after 1 dph, were exposed to different concentrations (2.5, 5, 10, 20 mg/L in ERM) of GO (Sigma-Aldrich, St. Louis, MO) for 96 h continuously by immersion. GO (2 mg/mL or 2000 mg/L, dispersed in water) was initially diluted to 20 mg/L (which is equivalent to 20 µg/mL; 100-fold) by ERM and sonicated in ice temperature for 5 min (2s on-1s off pulse, 225 w) using a probe sonicator (ultrasonicator LPX 750, Cole Palmer, Chicago, IL, USA). The sonicated GO (20 mg/L) was further diluted to desired concentrations (10.0, 5.0, 2.5 mg/L) by ERM. After sonication and required dilution, 1 ml of the GO in ERM (2.5, 5.0, 10.0, and 20.0 mg/L) was added directly to the fries and maintained them in a water bath at 25 ± 1 °C in a 13 × 75 mm glass tubes under 16 h light and 8 h dark light cycle. The controls (no GO) were maintained in identical conditions in ERM. The highest concentration of GO used in this study (20 mg/L) was based on a study conducted by Mullick Choudhury et al. [6] on graphene nanoribbons (GNRs) exposure experiment on medaka embryos. The sonicated nanomaterial was further checked by TEM at the facilities available at the Jackson State University, Jackson, MS, USA [2–3]. The fries were kept fasting (no exogenous food) during the exposure period. On 5 dph, survived fries/ larvae were transferred to BSS and maintained in 500 mL glass jars (4 fries/ 200 mL BSS) in a water bath at 25 ± 1 °C with 16L: 8D light cycles. Some of the fries were used for live imaging under an Olympus CKX53 inverted microscope attached with DP22 camera and cellSens software (Fig. 1a–e). During live imaging and photography, fries were anesthetized in MS 222 and returned to BSS for further maintenance until 47 dph. The mortality of the cultured fries/larvae was checked every day and the media changed once a week. The larvae (stages 42–43) were sacrificed after 42 days (47 dph) post-treatment. The length (cm) and weight (mg) of the larvae were recorded before sacrifice (Fig. 2a, b; ST1). The anterior parts of the body were preserved in 4% paraformaldehyde (PFA) in 20 mM PBS for histological evaluation of gonads (testis/ovary), liver, and kidney (Fig. 4a–f; Figs. 5 and 6); the post anal tails were preserved in 500 µL TRI reagent (Sigma-Aldrich, St. Louis, MO) and kept at -20 °C until genotyping the Y chromosome-specific male sex determining gene, *dmy* (Fig. 4h).

2.2. Histology of gonads, liver, and kidneys

The trunk region of the medaka (excluding head and tail), fixed in 4% PFA containing 20 mM PBS for 48 h (changed once after 24 h) followed by thoroughly washing in water. The washed tissues were dehydrated in graded alcohols (30–100%), cleared in xylene and embedded in paraffin (58–60 °C). Serial sections at 5 µm thickness were cut in a manual rotary microtome (Olympus cut 4055). The sections were used for hematoxylin and eosin (HE) staining. The photomicrographs of the sections were captured using an Olympus B-max 40 microscope attached

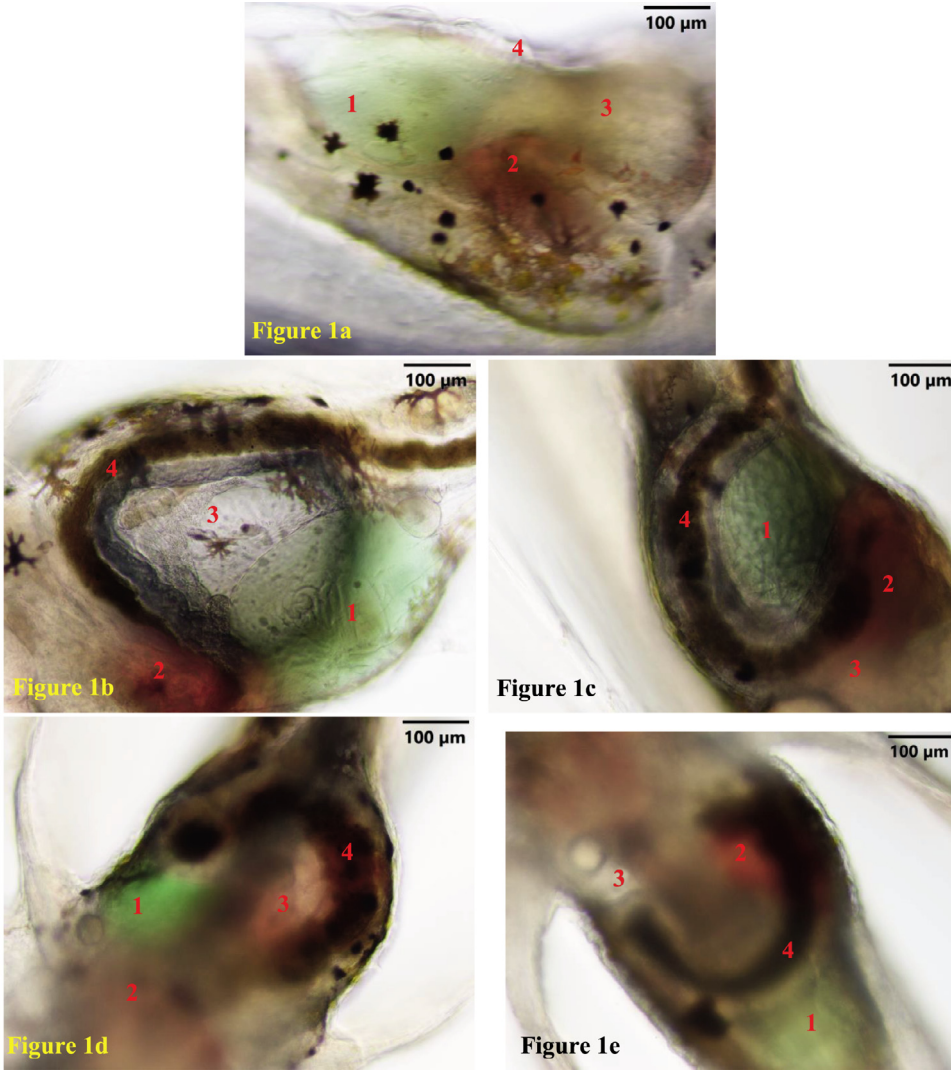


Fig. 1. Accumulation of GO into the body of the medaka fries/larvae after 96 h (4 consecutive days) exposure. Representative microscopic images of live medaka fries. Figures 1a = control, 1b = 2.5 mg/L, 1c = 5.0 mg/L, 1d = 10 mg/L and 1e = 20 mg/L. Numerical numbers used to identify gall bladder (1), spleen (2), yolk (3) and gut (4).

to a camera with Q-capture Pro 7 software (Media Cybernetics, Inc, Rockville, MD). Some of the photomicrographs were taken using Olympus CKX53 inverted microscope with DP22 camera and cellSens software (Huntoptics &Imaging, Pittsburg, PA, USA). Histopathological evaluation of the tissues (testis, ovary, liver, and kidneys) was done following OECD guidelines [1–3,7]. The digital images of testis and ovary (minimum three images in different regions of the organs) were further analyzed by imagej (http://www.imagej.nih.gov/ij) software and used for manual counting the spermatogonia (SPG) and primary spermatocytes (SPT) in the testis and stromal follicles (PNO and CAO) in the ovary (Fig. 4g; ST 2). The number of SPG or SPT present in a particular testicular lobule of males (both XY and XX genotypes; ST3 and ST4) or the ovarian

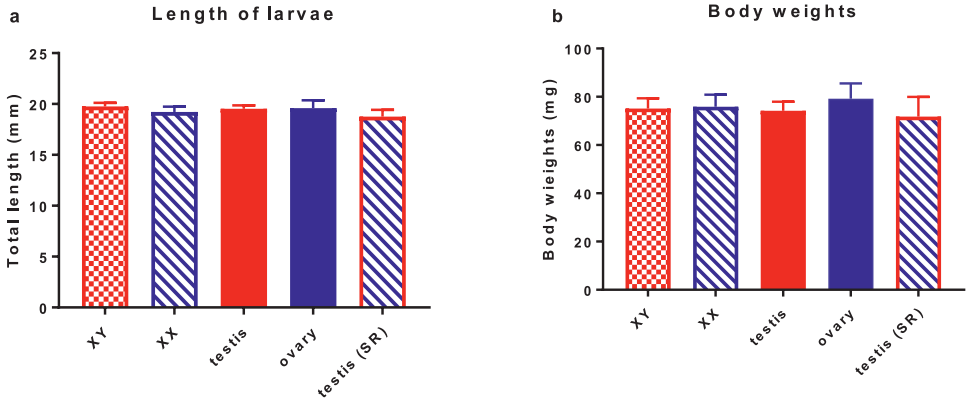


Fig. 2. The growth of the experimental fish (larvae) on 47th dph. The growth of the larvae was determined by measuring the total length (Fig. 2a) and weight before sacrificing the larvae. According to the phenotypic (testis and ovary) and genotypic classification (XY and XX) we found five categories of larvae. These larvae either had testis (n = 29) or ovary (n = 10) as gonads or XY genotypes with testis (n = 21) and XX genotypes with testis or ovary (n = 18). The sex-reversed (SR) larvae had testis even though they were genotypically XX (n = 8). During phenotypic evaluation, SR larvae were included in testis group (n = 29); however during genotypic evaluation SR larvae were included with XX genotypes (n = 18). SR= sex-reversed XX larvae with phenotypic testis.

follicles (PNO and CAO) in the ovary (XX genotype) were counted manually from the digital images (testis/ovary) and the area of a particular lobule (testis) or ovary was determined by imagej software. The data were expressed as number of SPG or SPT /mm^2 area of testis of that particular lobule (Fig. 4i–k; ST3, and ST4). For ovary, as there are only two types of countable follicles (PNO and CAO) found (both nonvitellogenic and vitellogenic oocytes were absent in the ovarian sections), we expressed the results in percent distribution either PNO or CAO [(PNOs X100/ (PNO+ CAO) or CAOX100/ (PNO+CAO)] found in that particular image of the ovary (Fig. 4j; ST 2).

2.3. Extraction of genomic DNA

The post anal tails preserved in TRI Reagent (Sigma-Aldrich, St. Louis, MO) and kept at –20 °C were used for genomic DNA extraction based on HotSHOT technique [4] with some modifications [1]. The tails were thawed at room temperature and carefully prepared for DNA extraction avoiding contamination or mixing of DNA with other tail DNA samples. Initially, each tail was washed in 1X PBS (Sigma-Aldrich, St. Louis, USA) separately, and a small piece was cut by a sharp disposable razor blade and transferred to a 2 ml centrifuge tube containing 1 ml PBS (1X). The tissue was washed in PBS (1 ml) twice by spinning for 10 min at 4 °C at 2500 rpm. Each washed tail piece was transferred to 200 µL PCR tubes containing 75 µL NaOH-EDTA (25 mM NaOH and 0.2 mM EDTA) and heated at 98 °C for 1 h in a Thermal cycler (Techne, Cole Palmer, Chicago, IL). After heating, the samples were cooled to 15 °C and 75 µL of 10 mM Tris-HCl (pH 5.5) were added, vortexed, followed by spinning for 10 min at 2500 rpm (Thermo-Fisher Scientific) at room temperature. Until genotyping, the supernatant portions were kept at -20 °C. The quality of the DNA was checked in 1% agarose gel containing ethidium bromide (0.01%) and the concentration was determined by a NanoDrop (Thermo-Fisher, St. Louis, MO). The 260/280 nm OD values (ranged from 1.15 to 2.13 with mean± SEM = 1.51 ± 0.04; n = 39) was used to infer DNA quality. Generally, 1 µL of the extracted DNA (631. 6 ± 81.25 ng/µL, n = 39) was used for PCR analysis [1].

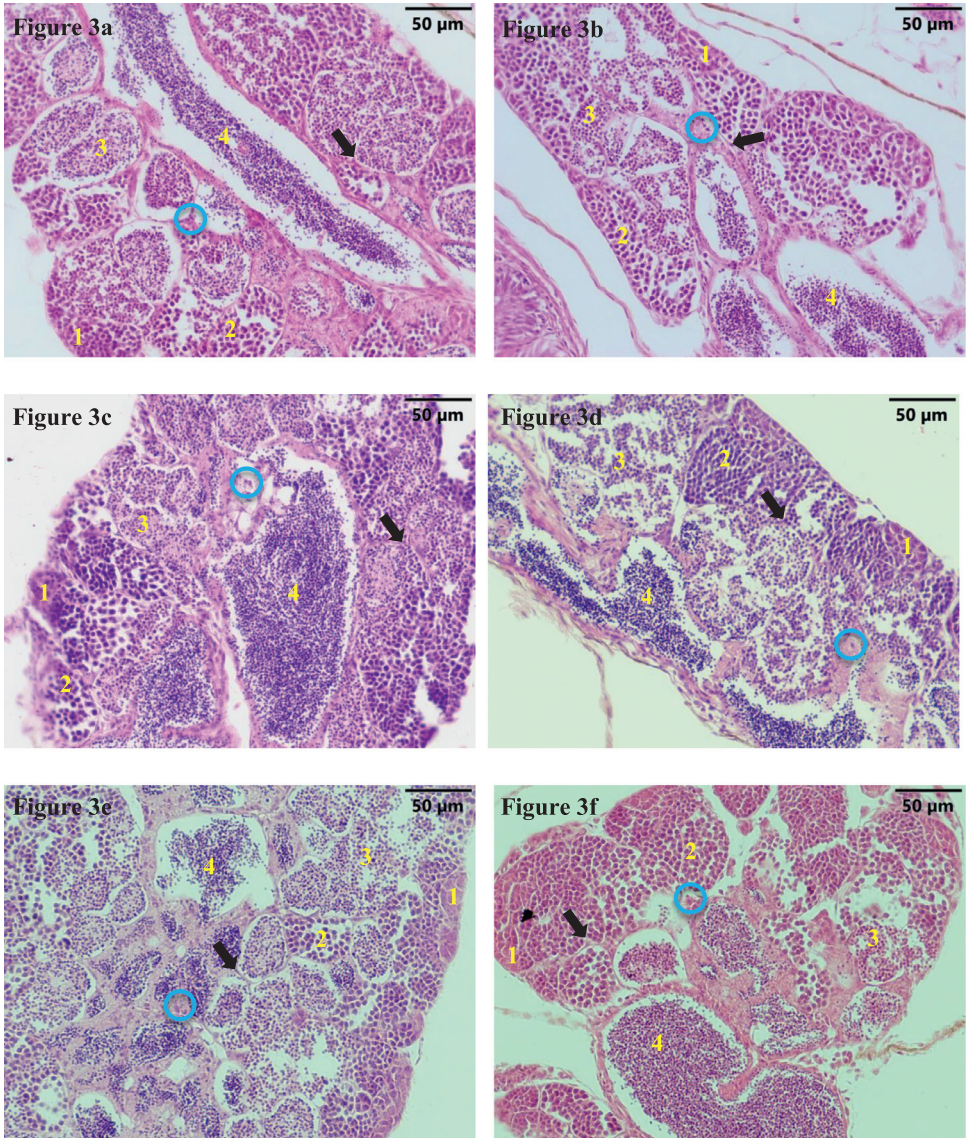
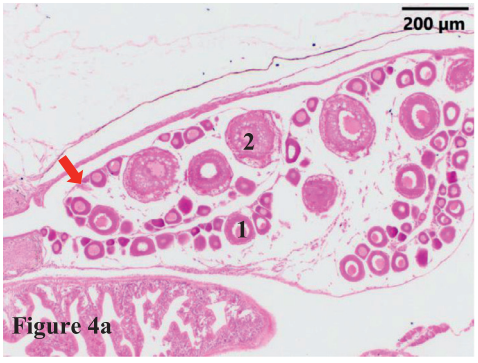
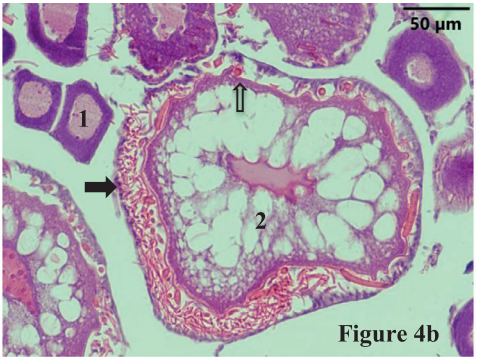
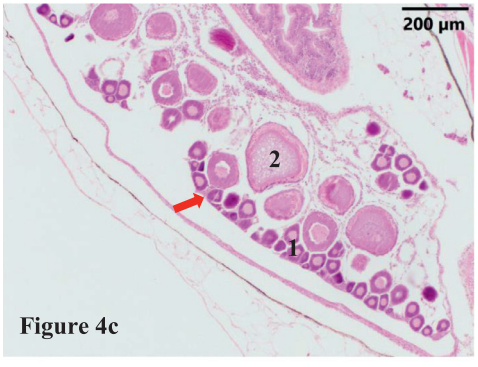
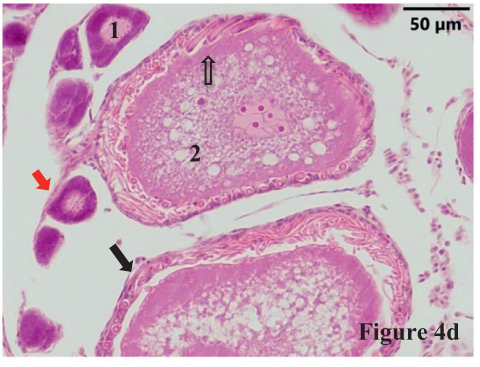


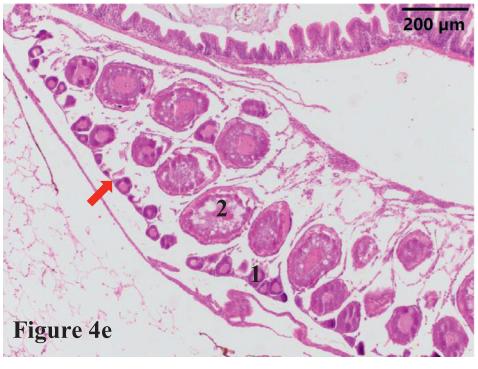
Fig. 3. Histopathological investigations on the effects of GO in the testis of Japanese medaka larvae. The fish with phenotypic testis had both XY (Fig. 3a, c and e) and XX (Fig. 3b, d and f) genotypes. Data indicated that both XY and XX testis had all the germinal components (labeled in numerical numbers; 1 = SPG, 2 = primary SPT, 3 = secondary SPT) and sperms (4) as well as Leydig cells (blue circles) and Sertoli cells (arrows in black). No ovo-testis or testis-ova like structures were observed either in XY (Fig. 3a, c and e) or in XX (Fig. 3b, d, or f) testis. Figure 3a (XY) and 3b (XX) are representative photomicrographs from control larvae (no GO); Figures 3c (XY) and 3d (XX) are from larvae exposed to 5.0 mg/L GO; Fig. 3e (XY) and f (XX) are from larvae exposed to 10 mg/L GO. The representative Figures of other two concentrations of GO (2.5 and 20 mg/L) used in this study were presented previously [1]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)











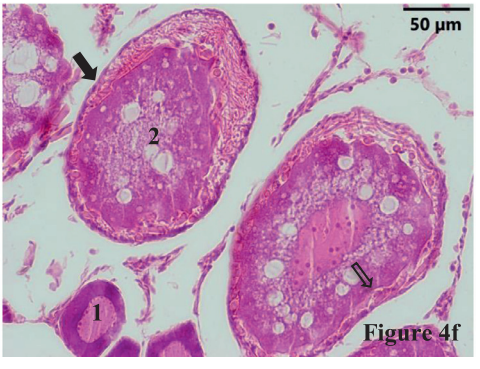


Fig. 4. Effects of GO exposure on ovarian development of Japanese medaka larvae. Representative photomicrographs of the ovaries of control (Fig. 4a and b), 5.0 mg/L GO (Fig. 4c and d) and 10.0 mg/L GO (Fig. 4e and f) are presented. The representative figures of other two concentrations of GO (2.5 and 20 mg/L) used in this study were presented previously [1]. Two types of follicles, PNOs (labelled as 1) and CAO (labelled as 2) were found in the stromal compartments of the ovary. The small red arrows in Fig. 4a, c, and e indicate the germinal epithelium. The chorion (empty arrows) and perifollicular cells (filled arrows) in CAO found in stage 1 ovaries are also presented (Fig. 4b,d, and f). The follicular data were presented in bar columns (Fig. 4g). Bar head with asterisks (*) indicate $p < 0.05$. Fig. 4g showed the distribution of stromal follicles (PNO and CAO) as % of ovary in two different stages of maturity (stage 0 and stage1). Bar heads with * indicate that the data are significantly different from the control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

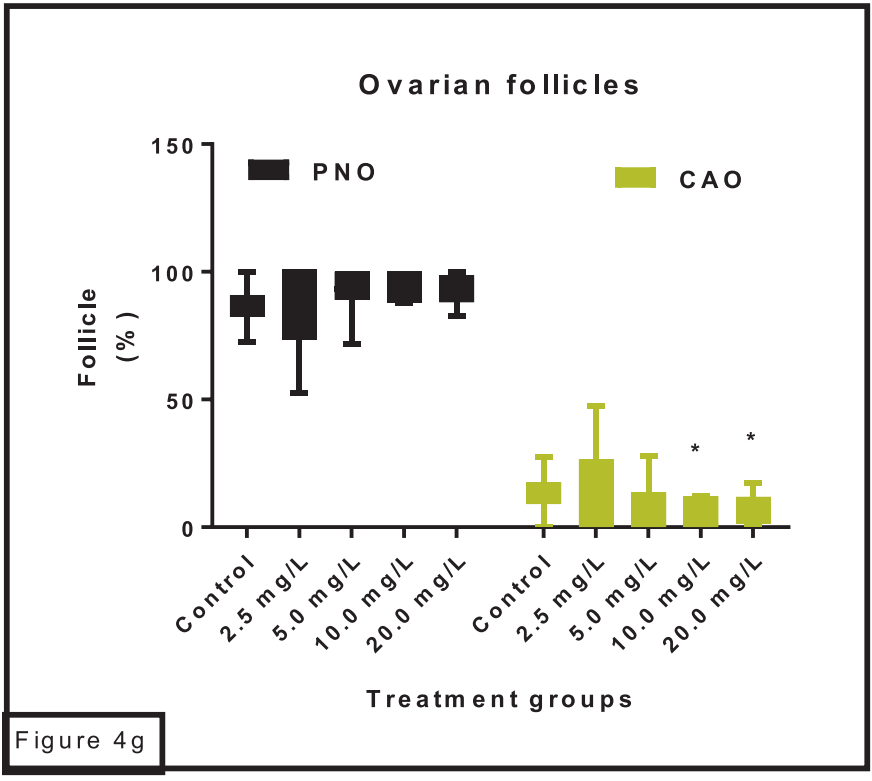


Fig. 4. Continued

2.4. Genotyping of the DNA

We used PCR-based methods for genotyping sex genes (*dmy*) of the larvae (Fig. 4h) [1]. One µL of the extracted DNA in a 20 µL final reaction volume was used to amplify a *Y* chromosome-specific male sex determining gene *dmy* [8] or *dmrt1* [9] using two different sets of buffers and primers. The *dmrt1bY* gene of Japanese medaka is a duplicated version of autosomal *dmrt1a* gene, sharing high similarity (sequence identity 94.5% and amino acid similarity of 89.4% over the entire coding region [10], even in introns [11]. The first set of primers (F1) (Forward: 5′-TTG AAG ACC CCG CTG G-3′), (R1) (reverse: 5′-CGG CCC AAA TTC TGG CAT CTT TGC-3′) targets *dmy* (*dmart1Y*; GenBank Locus AY129241) producing a PCR product of 355bp in male genotypes (XY) and no amplification in fish with female (XX) genotype [12]. The Taq PCR master mix kit (Qiagen, Germantown, MD) was used for the product (355 bp) amplification. The second set of primers (F2 and R2) (F2: 5′- CCG GGT GCC CAA GTG CTC CCG CTG-3′, R2: 5′-GAT CGT CCC TCC ACA GAC AAG AGA-3′) [13]. These primers (F2 and R2) amplified two products for male genotype (XY) one at 986 bp targeting male specific *dmy* (*dmrt1bY*) gene and a second product at 1249 bp targeting autosomal *dmtr1a* gene (GenBank Locus AY157712); however, for females (XX) a single product at 1249 bp targeting *dmy1a* gene was observed [1,13,14]. The multiplex PCR kit (Qiagen, Germantown, MD) was used for product amplification. The reaction mixture contained 10 µL of 2x master mix (Taq PCR buffer for first set of primers and the multiplex PCR buffer for the second set of primers) 50 pM forward and reverse primers, 1 µL extracted DNA (631. 6 ± 81.25 ng/µL) and the volume was adjusted to 20 µL with nuclease-free water. The amplification reaction was conducted in a Thermal cycler (Techne, Cole Palmer, Chicago, IL)

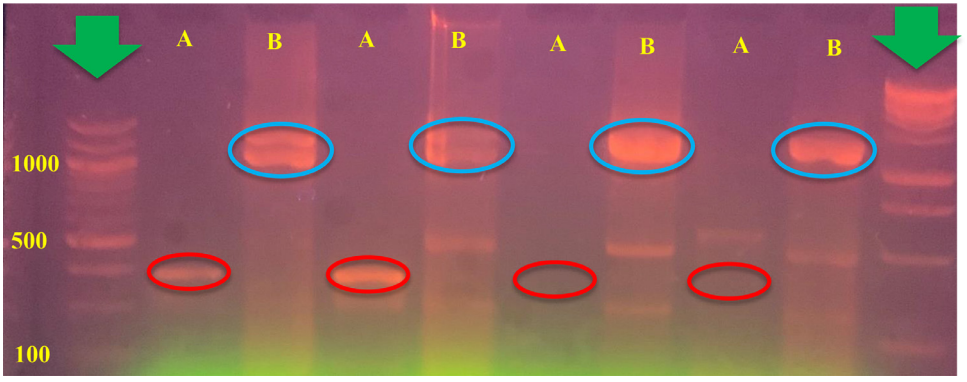


Fig. 4h. Representative gel picture of tail DNA amplification products of Japanese medaka larvae (47 dph) used in genotyping the male sex determining gene (*dmy*) is presented. The post anal tail was preserved in TRI reagent and kept at -20 °C until DNA extraction. The DNA, after 2X washing in PBS, was extracted by digesting the tail tissue in sodium hydroxide (25 mM)-EDTA (0.2 mM) solution at 98 °C for 1 h followed by neutralization with 40 mM Tris-HCL (HotSHOT)[3]. The DNA was amplified with 2 sets of primers with two different PCR buffers. The amplified products were separated in 2% agarose gel electrophoresis containing 0.01% ethidium bromide, and the gels were viewed on an UV-illuminator. The photographs were taken in a smartphone digital camera. Lanes A represent the amplified product of primer set 1 (F1, R1)(F1 forward = 5′- TTG AAG ACC CCG CTG G -3′, R1 reverse 5′- CGG CCC AAA TTC TGG CAT CTT TGC -3′) using PCR buffer (red circle) and lanes B represent the amplified products of the primer set 2 (F2, R2) (F2 forward = 5′- CCG GGT GCC CAA GTG CTC CCG CTG-3′, R2 reverse = 5′- GAT CGT CCC TCC ACA GAG AAG AGA-3′) using multiplex buffer (blue circle) of the tail DNA extracted from the same fish. The amplification reaction in a 20 µL volume containing 1 µL of extracted DNA (631.6 ± 81.25 ng/µL) was conducted in a thermal cycler (Technie, Cole Parmer, Chicago, IL) with the following reaction conditions: initial denaturation 3 min, followed by 40 cycles of denaturation 0.5 min at 94 °C, annealing 1 min at 57 °C, and extension 2 min at 72 °C, followed by a final extension 7 min at 72 °C for primer set 1 (lanes A; F1, R1). For the second sets of primers (lanes B; F2, R2), reaction conditions are, initial denaturation 15 min (hot start), followed by 40 cycles of denaturation 0.5 min at 94 °C, annealing 1.5 min at 57 °C, and extension 2 min at 72 °C, followed by a final extension of 10 min at 72 °C. A lanes showed a 355 bp amplified product only in XY genotypes and no amplification in XX genotypes (red circle). B lanes showed two bands (1249 and 986 bp) for XY and one band (1249 bp) for XX genotypes (blue circles). The arrows in green indicate 100 bp ladder. A few of the DNA fragment sizes (bp) in 100 bp ladder (yellow) are marked by numbers (1500 bp, 100 bp and 300 bp). The buffers used in PCR reactions (Taq buffer and multiplex buffer) were obtained from Qiagen (http://www.qiagen.com). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

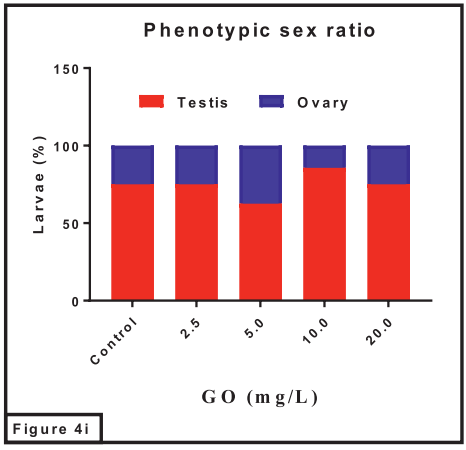
at 95 °C for 3 min for Taq buffer samples (primer sets F1R1) and 15 min for multiplex buffer samples followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 57 °C for 60 s, extension at 72 °C for 2 min and a final extension at 72 °C for 7–10 min. The PCR products were separated in 2% agarose gel containing 0.01% ethidium bromide in TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, pH 8.3). The gels were viewed in an UV illuminator and the photographs were taken in a smartphone digital camera. The genetic sex of the fish was determined visually by viewing the gel pictures or the photographs (Fig. 4h).

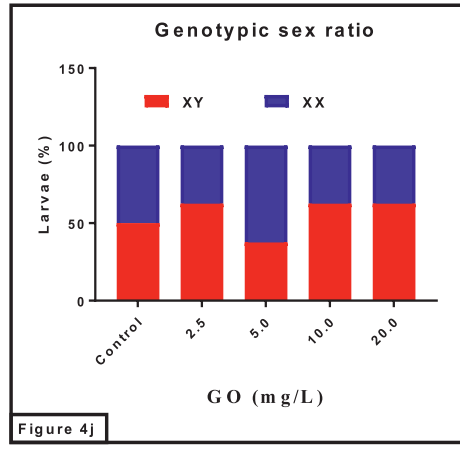
2.5. Statistical analysis

All statistical analysis was conducted using SPSS22.0 (IBM corporation) and GaphPAD prism 7.04 (GraphPad Prism, San Diego, CA).

2.6. Larval growth (lengths and weights)

We used descriptive analysis to evaluate the mean lengths and weights of all the larvae. The normality of the data (lengths and weights) were determined by D'Agostino-Pearson (DP) or Shapiro-wilk's (SK) test. Statistical significance was defined as $p \leq 0.05$. Both DP and SK test





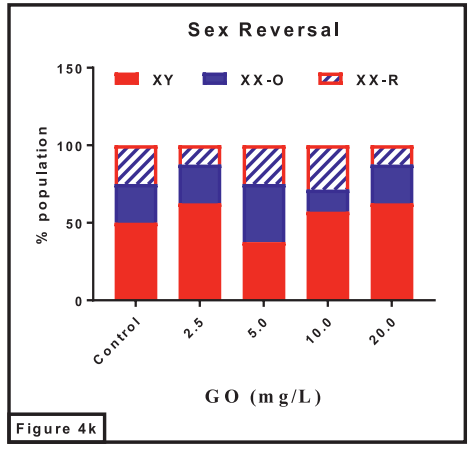


Fig. 4i–k. Graphical representation of the phenotypic and the genotypic sex ratios as well as sex reversal in Japanese medaka larvae observed at 47 dph. The phenotypic sex of the larvae was determined by gonad histology (Fig. 4i), genotypic sex by genotyping male sex-specific gene *dmy* (Fig. 4j), and sex reversal in XX genotypes (Fig. 4k) was estimated after viewing either testis or ovary as phenotypic gonads (Figs. 3 and 4).

indicated that the data were normally distributed except for the lengths of larvae exposed to 2.5 mg/L GO ($p = 0.04$) which is significant. With this exception, we assumed that the data were normally distributed and meet the percepts for using a parametric test (normality and homoscedasticity) for multiple comparisons. We used one-way ANOVA followed by post-hoc Tukey's multiple comparison test or unpaired parametric "t" test including Welch's correction and expressed the data as mean ± SEM.

2.7. Histological evaluation of gonads (ovary and testis)

For the data analysis referring to histological parameters (stromal follicles in the ovary, SPGs and SPTs in the testis) we performed the Kruskal-Wallis test once that data did not meet the criteria of using a parametric test (normality and homoscedasticity). Mann-Whitney's test (non-parametric tests) were used as a post hoc test and the level of significance was considered at $p \leq 0.05$.

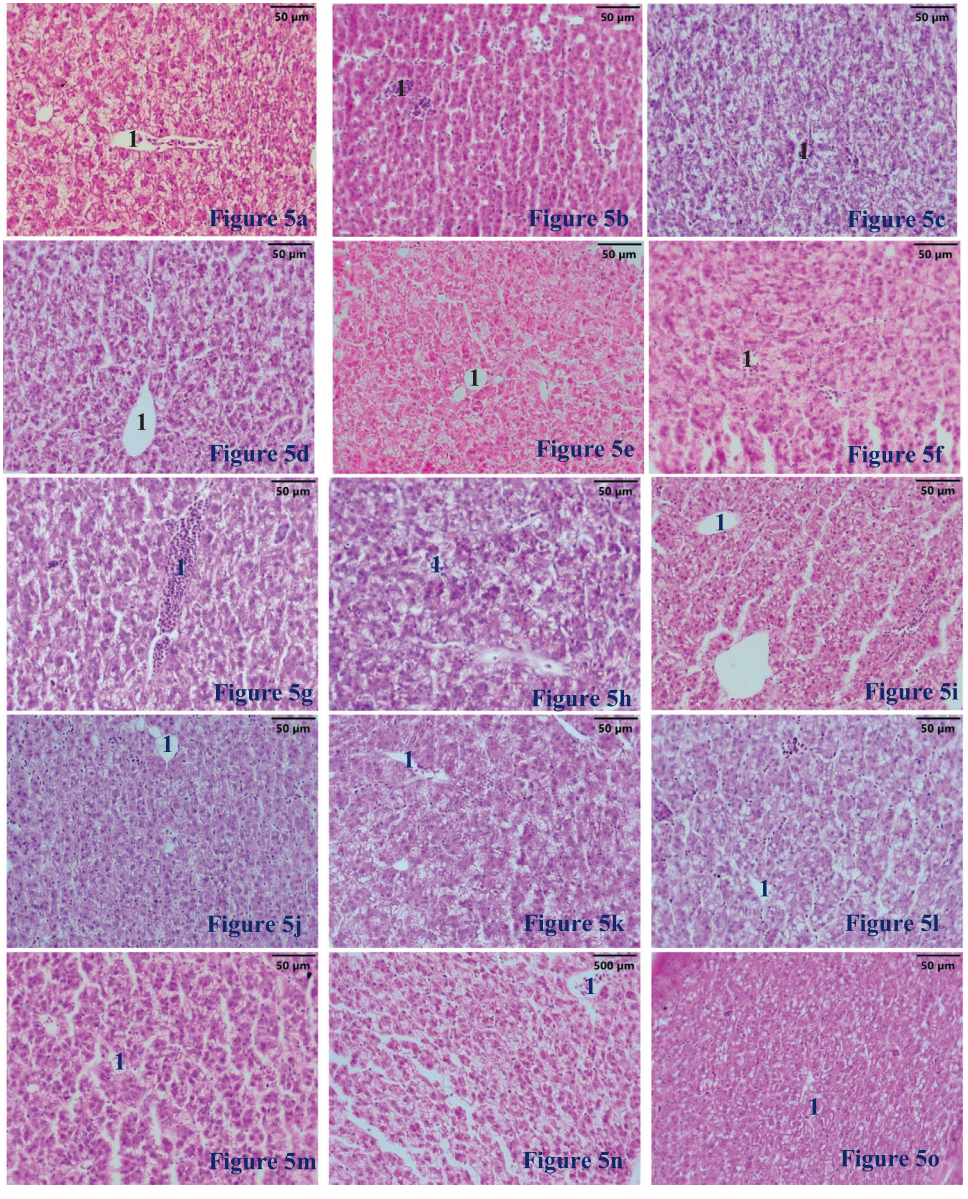


Fig. 5. Effect of GO on the histopathology of the liver of Japanese medaka larvae at 47 dph. Representative photomicrographs of the histopathology of the liver tissue of XY males (Fig. 5a,d,g,j and m), XX males (Fig. 5b,e,h,k and n) and XX females (Fig. 5c,f,i,l and o) were evaluated using HE staining. Numerical number 1 = Sinusoidal cavity. Photomicrographs 5a (control), 5d (exposed to 2.5 mg/L GO), 5g (exposed to 5.0 mg/L GO), 5j (exposed to 10.0 mg/L) and 5m are from males (testis) with XY genotypes. Photomicrographs 5b (contol), 5e (exposed to 2.5 mg/L GO), 5h (exposed to 5.0 mg/L GO), 5k (exposed to 10.0 mg/L GO) and 5n (exposed to 20.0 mg/L GO) are from males (testis) with XX genotypes; Fig. 5c (control), 5f (exposed to 2.5 mg/L GO), 5i (exposed to 5.0 mg/L GO), 5l (exposed to 10.0 mg/L GO) and 5o (exposed to 20.0 mg/L) are from female (ovary) larvae with XX genotypes.

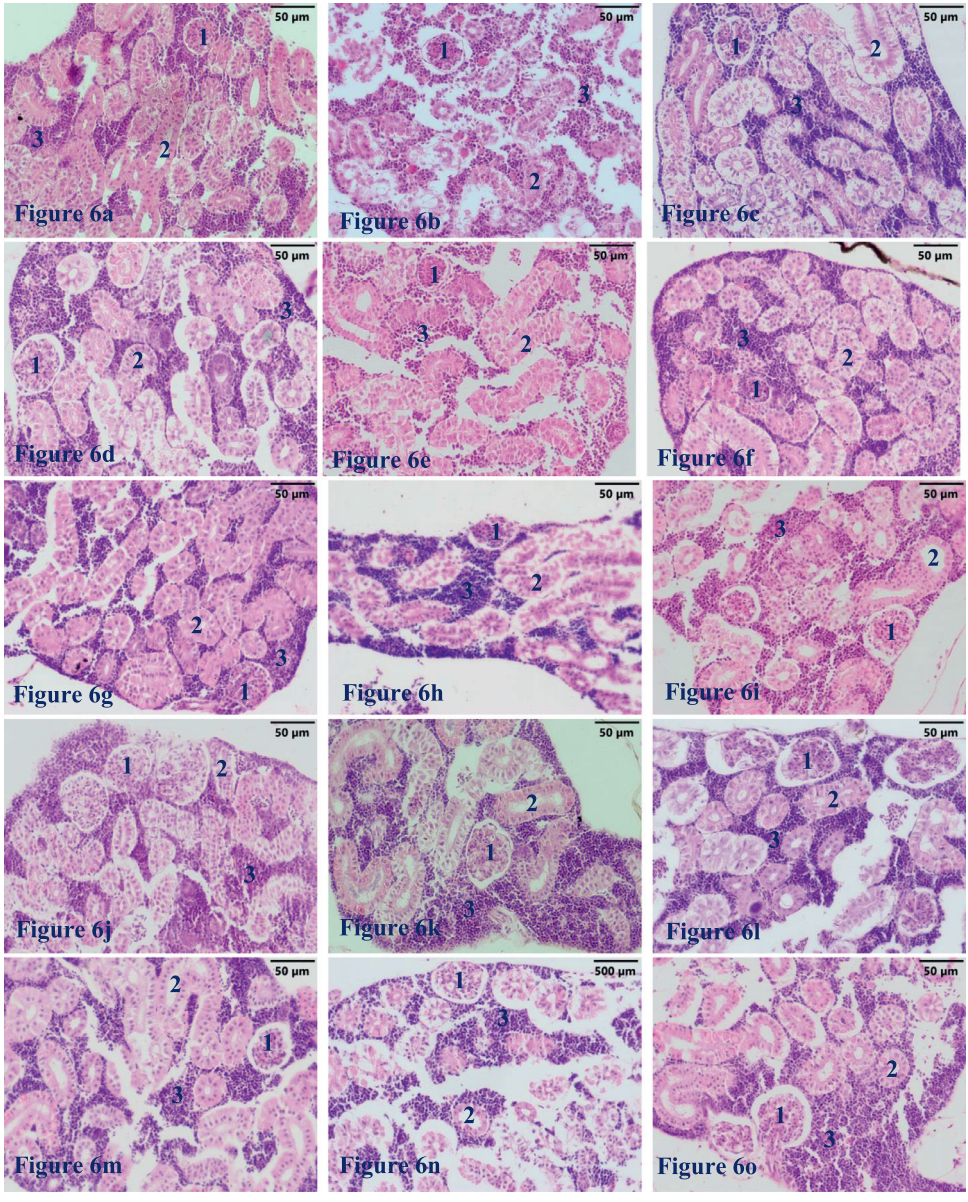


Fig. 6. Effect of GO on the histopathology of the kidney of Japanese medaka larvae at 47 dph. Representative photomicrographs of the histopathology of the kidney tissue of XY males (Fig. 6a,d,g,j,m and g), XX males (Fig. 6b,e,h,k and n) and XX females (Fig. 6c,f,i,l and o) were evaluated using HE staining. Numerical number 1 = glomerulus; 2 = renal tubules; 3 = interstitial lymphomymetic cells. Photomicrographs 6a (control), 6d (exposed to 2.5 mg/L GO), 6g (exposed to 5.0 mg/L GO), 6j (exposed to 10.0 mg/L GO) and 6m (exposed to 20 mg/L GO) are from males (testis) with XY genotypes; photomicrographs 6b (contol), 6e (exposed to 2.5 mg/L GO), 6h (exposed to 5.0 mg/L GO),6k (exposed to 10.0 mg/L GO), and 6n (exposed to 20 mg/L GO) are from males (testis) with XX genotypes; photomicrographs 6c (control), 6f (exposed to 2.5 mg/L GO), 6i (exposed to 5.0 mg/L GO), 6l (exposed to 10.0 mg/L GO) and 6o (exposed to 20.0 mg/L GO) are from female (ovary) larvae with XX genotypes.

Ethics statement

No human subjects were involved in this research.

The Institutional Animal Care and Use Committee (IACUC) of the Jackson State University, Jackson, MS, USA, approved all experimental protocols.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships which have or could be perceived to have influenced the work reported in this article.

CRediT Author Statement

Anitha Myla: Formal analysis, Visualization, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing; **Asok K. Dasmahapatra:** Conceptualization, Formal analysis, Visualization, Investigation, Methodology, Project administration, Software, Supervision, Writing – original draft, Writing – review & editing; **Paul B. Tchounwou:** Conceptualization, Funding acquisition, Visualization, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.dib.2021.107330.

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