Exposure of zebrafish to environmentally relevant concentrations of mercury during early life stages impairs subsequent reproduction in adults but can be recovered in offspring

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ABSTRACT

Mercury (Hg) is a global pollutant that poses potential threats to health of fishes. Although effects of Hg on reproduction of fishes have been documented, little is known about effects of exposure to Hg\textsuperscript{2+} during early life stages on subsequent reproductive fitness of adults or whether these effects can be transferred to offspring. In this study, zebrafish embryos were exposed to environmentally relevant concentrations of Hg\textsuperscript{2+} (0.6, 3 or 15 \(\mu\)g/L) for 5 days and then depurated in clean water for another 115 days. Exposure to Hg\textsuperscript{2+} during early life stages disturbed the balance of sex hormones and gametogenesis by altering expression of mRNA for genes involved in the hypothalamic-pituitary-gonadal axis, which resulted in delayed gonadal development and lesser gonado-somatic index, thereby resulting in lesser fecundity. A similar, but less pronounced effect was observed in F\textsubscript{1} females that were not exposed directly to Hg, whereas such damage was neither observed in F\textsubscript{1} males nor either sex during the F\textsubscript{2} generation. Exposure to Hg\textsuperscript{2+} during early life can impair subsequent reproduction in adults and has intergenerational effects on F\textsubscript{1} females, but this reproductive damage can be recovered in F\textsubscript{1} males and in F\textsubscript{2} females.

1. Introduction

Mercury (Hg) is one of the most dangerous contaminants in aquatic environments. Due to natural and anthropogenic processes and activities, Hg has been released into the atmosphere and into aquatic environments. Thus, current concentrations of Hg in the environment are greater than historical backgrounds in most areas, which has raised concern around the world (Driscoll et al., 2013; Zhang et al., 2018). Hg enters aquatic organisms mainly through diet and across gills (Morcillo et al., 2017) and can easily pass through biological membranes and accumulate, which can then damage various organs and diminish health (Korbas et al., 2008; Morcillo et al., 2017). In comparison to other aquatic organisms at lower trophic levels (such as plankton and invertebrates), fishes are more vulnerable to effects of Hg due to bio-accumulation and biomagnification through food chains. In the aquatic environment, Hg presents in various forms as elemental Hg (Hg\textsuperscript{0}), organic compounds (such as methylmercury, MeHg), and inorganic compounds (such as Hg\textsuperscript{2+}) (Farina et al., 2013). Some processes including microbial transformations and photochemical reactions are responsible for converting elemental Hg into inorganic and organic Hg (Devlin, 2006). MeHg is the most toxic form and has received more attention, but information on long-term and intergenerational effects of inorganic Hg, a major form in industrial, pharmaceutical and commercial activities, on fish is scarce.

As a critical period of development, early life stages are important to fish population dynamics (Huang et al., 2010) and during early life stages, fish are particularly sensitive to some pollutants, including Hg (Korbas et al., 2011). Toxicity tests during critical, early windows of development of fishes are used to assess whole organism responses (U.S. EPA OPPTS 850.1400, 1996) and results of these short-term assays are often used to predict acute toxicity to juvenile, adult and older stages of development (Lammer et al., 2009). Increasing evidence has emerged...
that short-term exposures to environmental contaminants during early life stages of fishes can affect fitness later in life, even if the exposure does not continue into those later stages of development (Jonsson and Jonsson, 2014). Nevertheless, little is known about impacts of exposure to Hg\(^{2+}\) during early development on subsequent outcomes or lasting effects on future generations.

In fishes, reproduction is a complex process primarily modulated by the hypothalamic-pituitary-gonadal axis (HPG-axis) and steriodogenesis including production of 11-ketotestosterone (11-KT) and 17\(^{-}\)-estradiol (E2) (Yaron and Levavi-Sivan, 2011). Moreover, some key regulatory factors involved in cell proliferation, such as PCNA, cyclin B1 and D1, and meiosis, such as Sycp3 and Dmc1 are particularly important for regulating spermatogenesis and oogenesis, which is essential to ensure reproductive fitness (Nagahama and Yamashita, 2008; Lan et al., 2020).

Impaired reproductive capacity can be observed due to lesser concentrations of gonadotropins and sex hormones, along with changes in expressions of mRNA for genes involved in synthesis of steroid hormones and gametogenesis (Wang et al., 2016). Developmental damage including mortality and malformations as well as lesser hatching success were observed after exposure of zebrafish (Danio rerio) embryos to Hg\(^{2+}\) (Zhang et al., 2016a). Exposure of adult zebrafish to Hg\(^{2+}\) resulted in impaired reproductive performance, characterized by histopathological changes and disturbances of reproductive hormones (Zhang et al., 2016b). The key question assessed in the study, results of which are presented here, is whether reproduction of adults is compromised due to exposure to transient concentrations of Hg during early life stages, and if so, whether the damage can be reversed and function recovered in offspring.

The zebrafish is used as a vertebrate model for toxicology studies because it possesses unique features, such as ease of culture, large reproductive capacity, transparent embryos and rapid organogenesis (Scholz et al., 2008). The purpose of this study was to evaluate effects of exposure to Hg\(^{2+}\) during early life stages of zebrafish on reproduction and to determine whether these effects can be carried over to subsequent generations. Normally developing embryos (2 h post-fertilization, 2 hpf) were exposed to environmentally relevant concentrations of Hg\(^{2+}\) (0.06, 0.6, 3 and 15 \(\mu\)g/L) for 5 days, and then grown-out in clean water until sexual maturity (120 days post-fertilization, 120 dpf). Histology of gonads, concentrations of sex hormones and expression of mRNA for genes involved in the HPG-axis were evaluated, and fecundity was assessed by use of a 21-day breeding study. In order to investigate intergenerational effects on reproduction, the same parameters measured in the F\(_0\) generation were also measured during the F\(_1\) and F\(_2\) generations. These findings not only facilitated understanding of mechanisms by which exposure to Hg during early life stages influenced reproduction in later life stages, but also helped to assess potential and persistent threats of Hg to populations of fishes.

2. Materials and methods

2.1. Chemicals

Mercuric chloride (HgCl\(_2\), \(\geq\)99.5 %) and other chemicals were purchased from Sinopharm Group Corporation (Shanghai, China). E2 and 11-KT assay kits were obtained from Cayman Chemical (Ann Arbor, USA). RNAiso Plus, PrimeScript\(^{TM}\) RT and SYBR\(^{TM}\) Premix Ex Taq\(^{TM}\) reagent Kits were from TaKaRa (Dalian, China).

2.2. Fish and treatments

This study was approved by the Animal Care and Use Committee of Key Laboratory of Animal Biology of Chongqing at Chongqing Normal University (Permit: Zhao-20171125–01). Zebrafish embryos were obtained and maintained in accordance with previously described methods (Zhang et al., 2016a). Briefly, adult female and male zebrafish (wild-type AB strain) were separated before spawning and fertilized eggs were collected when the light was turned on in the morning. Normally developing embryos (2 hpf) were randomly distributed into polypropylene boxes containing 1 L of HgCl\(_2\) solutions (0, 0.6, 3 or 15 \(\mu\)g Hg\(^{2+}\)/L). There were 3 replicates for each treatment, and each box included 150 embryos. Exposure was performed during the period from 2 hpf to 5 dpf. Concentrations of Hg used in this study were based on results of a dose-range finding pilot experiment which showed that 15 \(\mu\)g Hg\(^{2+}\)/L could impair embryonic development and increase the mortality of embryo or larvae. Moreover, these concentrations were close to those reported to occur in the water environment. For example, the greatest concentration of total Hg (tHg) detected in water from the Tianshan Mountains in China, the Carson River in the USA, the Wanshan mining area in China, and the Obuasi mining area in Ghana was 1.75 (Zhao et al., 2015), 2.1 (Bonzongo et al., 1996), 10.58 (Horvat et al., 2003), and 25.3 (Armah et al., 2010) \(\mu\)g/L, respectively. Exposure solutions were sampled on the last day of exposure, and measured concentrations of tHg were 0.06 \(\pm\) 0.01, 0.54 \(\pm\) 0.05, 2.50 \(\pm\) 0.27 and 13.43 \(\pm\) 0.53 \(\mu\)g/L for nominal concentrations of 0, 0.6, 3 and 15 \(\mu\)g/L, respectively. Eggs were incubated at a constant temperature of 28 \(\pm\) 1°C and a light regime of 14 h L/10 h D. Dead embryos or larvae were counted and removed every 12 h, and the cumulative mortality at the end of exposure (5 dpf) was 5.16 \(\pm\) 2.02, 6.9 \(\pm\) 1.20, 10.59 \(\pm\) 3.15 and 20.55 \(\pm\) 6.47 % for the control, 0.6, 3 and 15 \(\mu\)g Hg\(^{2+}\)/L groups, respectively. Half of solutions were replaced daily with fresh solution containing corresponding concentrations of Hg\(^{2+}\) (wastewater was collected and properly disposed of). At 6 dpf, all larvae were transferred to clean water and fed until adulthood (120 dpf, referred to as F\(_0\) generation). From 6–20 dpf, larvae were fed live Paramecia three times daily, then fed live Paramecia and Artemia from 21 to 50 dpf. Fish were fed live Artemia and commercial flake diet after 50 dpf. After sex of individuals could be distinguished by phenotype, females and males in each tank were cultured separately. Water quality was monitored throughout the experiment for: temperature, 27.8–28.6 \(^\circ\)C; hardness, 127.01–136.77 mm/L as CaCO\(_3\); dissolved oxygen, 7.27–7.64 mg/L; pH, 7.4–7.7.

2.3. Sample collections

At 120 dpf, fish were not fed for 24 h then anaesthetized with 0.01 % MS-222, except for 3 males and 3 females left randomly in each replicate tank (a total of 9 fish per sex for each treatment) to assess reproductive performance. Afterwards, blood from 25 fish of the same sex per tank was collected and pooled as one sample (3 replicate samples for each treatment) for quantification of hormones in plasma, after body mass had been determined. Individual fish were then dissected to acquire gonads and weighed to calculate the gonado-somatic index (GSI). Samples of gonads from 3 females and 3 males in each tank (a total of 9 fish per sex for each treatment) were used for histological examination, while another 2 males per tank (a total of 6 fish for each treatment) were used for sperm counting. Additionally, brains and gonads from 3 individual fish of the same sex per tank were pooled as one sample (3 replicate samples for each treatment) and homogenized in 1 mL ice-cold RNAiso Plus reagent to extract total RNA for real-time PCR (RT-qPCR) analysis. The rest of gonad samples were immediately immersed in liquid nitrogen and then stored at \(-80 \, ^\circ\)C until tHg quantifications or other analyses.

2.4. Reproductive assessments

Three females and 3 males from each replicate tank (a total of 9 fish for each treatment) were used in a 21-d breeding study. Briefly, 3 females and 3 males from a replicate tank were placed in a test tank (length 29 cm, width 21 cm, height 15 cm) with about 6 L water (27.7–28.5 \(^\circ\)C). A transparent plastic tray (length 10 cm, width 10 cm, height 1 cm) with screens and artificial aquatic plants was placed in the test tank as spawning area. The number of eggs spawned within 2 h of the beginning of the light period in the test tank was counted every
morning. During the 21-d breeding study, fish were maintained in the test tank and fed live *Artemia* and commercial flake diet three times daily. Fecundity was reported as cumulative eggs produced by 3 females in each test tank. Meanwhile, obtained embryos were used in the following study (see section 2.5).

2.5. Sample collections and reproductive assessments in offspring

Embryos produced by 3 paired parents of F<sub>1</sub> per tank were referred to as F<sub>1</sub> generation. 450 fertilized and normal embryos (3 replicates of 150 embryos) were selected and cultivated in clean, de-chlorinated water (no Hg<sup>2+</sup> added) until 120 dpf. Remaining embryos were cultured in clean water until 5 dpf for quantification of tHg. Similarly, embryos from 3 paired parents of F<sub>2</sub> per tank, hereafter referred to as F<sub>2</sub> generation were collected. Culture conditions, sample collections and reproductive assessments were the same as F<sub>1</sub> generation as mentioned above. The overall experimental design of this study is shown in Fig. 1.

2.6. Sample analysis

2.6.1. Quantification of tHg

Concentrations of tHg in exposure solutions were determined by atomic fluorescence spectrophotometer (PF6–3, PERSEE General Instrument Co. Ltd, Beijing, China) following the Environmental Protection Standards of People’s Republic of China (HJ 694–2014). Concentrations of Hg in larvae and gonads were quantified by use of a direct Hg analyzer (DMA-90, Milestone, Italy). For quality assurances, every 20 samples included a sample of standard reference materials (GBW 10050, National Center for Standard Reference Materials of China), a method blank, and three replicate samples. Concentrations of tHg in larvae or gonads were presented in ng/g wet weight (ng/g w.w.).

2.6.2. Histological examination

Ovaries and testes were fixed in Bouin’s fluid for 24 h and then were dehydrated in graded concentrations of ethanol. After being embedded in paraffin, samples were sliced to sagittal sections (5 μm). Specimens were stained by use of hematoxylin and eosin, and then were sealed with neutral gum. Finally, sections were scanned with Panoramic MIDI scanner (Budapest, Hungary) for histological examination. For ovarian sections, follicles were grouped as following stages: primary growth (PG), previtellogenic (PV), early vitellogenic (EV), midvitellogenic (MV) and full grown (FG) follicles (Li et al., 2011). Numbers and proportions of follicles at different stages of development in sections of ovaries were counted and calculated.

2.6.3. Sperm counting

Counting of sperm cells was performed according to methods outlined by Pradhan and Olsson (2015) with slight modifications. Briefly, dissected testes were submerged in 1 mL normal saline solution (0.9 %) and smashed by use of a scalpel and needle tubing. Samples were diluted an appropriate multiple and loaded into a 0.1 mm<sup>3</sup> volumetric hemocytometer. After allowing sperm to settle 3–5 min, five squares including four corners and a middle were counted by use of an optical microscope (UY2031, UOP Photoelectric, Chongqing, China) at 20× magnification.

2.6.4. Quantification of steroid sex hormones

Blood of the same sex fish in each tank was collected and pooled as one composite sample in a heparinized microfuge tube. Samples were centrifuged (5000 ×g, 10 min) and supernatants were obtained. Plasma 11-KT and E2 were measured by use of ELISA kits according to the manufacturer’s instructions. Coefficients of variation for intra- and inter-assay were less than 10 %.

2.6.5. Extraction RNA and quantitative RT-qPCR analysis

Total RNA was extracted from samples of brains, ovaries and testes by use of RNAsio Plus, and its quality was evaluated by a spectrophotometer (Nanodrop ND-2000, Thermo Fisher Scientific, USA) and agarose gel electrophoresis. RT-qPCR analysis was carried out as described previously (Xie et al., 2019). Briefly, 1 μg total RNA was used to synthesize cDNA by use of PrimeScript<sup>™</sup> RT reagent Kits. RT-qPCR amplifications with SYBR<sup>™</sup> Premix Ex Taq<sup>™</sup> reagent Kits and primers were used to quantify mRNA of all target genes. Specific primers used for each gene are provided in Supplementary Table S1. Thermocycling

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Fig. 1. Schematic diagram of the experimental design for this study, including the time points at which fecundity was measured and samples were collected. For each generation, there were 3 replicate tanks per treatment and each replicate contained 150 embryos. At each sampling time, 9 males and 9 females from each treatment (3 males and 3 females per replicate) were used for breeding study, and brains, gonads and blood of other fish were sampled for analyses.

Fig. 2. Concentrations of total mercury (tHg) in fish exposed for 5 days during early life stages to various concentrations of Hg<sup>2+</sup>. (A), F<sub>0</sub> generation; (B), F<sub>1</sub> generation; (C), F<sub>2</sub> generation. Data are mean ± SD of three replicate samples (each replicate contained 10 individuals for gonads and 100 individuals for larvae). * significant differences from the control at p < 0.05.
protocols were: 30 s at 95 °C; 40 cycles of 5 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Expression of mRNA for elongation factor 1-alpha (ef1α) was stable and used as a housekeeping gene. Expressions of mRNA of genes were normalized to ef1α by use of the 2−ΔΔCt method (Livak and Schmittgen, 2001).

2.7. Statistical analyses

Statistical analyses were conducted by use of SPSS 17.0 software and data were expressed as mean ± SD. The Kolmogorov–Smirnov test and Levene’s test were employed, respectively, to examine for normality of the data and the homogeneity of variances. If necessary, to meet the assumptions of parametric statistical tests, data were log-transformed. Statistically significant differences were determined by use of one-way ANOVA with Tukey’s multiple range test. Significant differences were taken as p < 0.05.

3. Results

3.1. Concentrations of tHg

In the F0 generation, concentrations of tHg were 5.48 ± 0.37, 5.82 ± 0.28, 6.71 ± 0.91 and 13.08 ± 3.10 ng/g w.w. in ovaries of female fish exposed to 0, 0.6, 3 or 15 μg Hg²⁺/L, respectively, and were 5.26 ± 0.21, 5.47 ± 0.19, 6.28 ± 0.46 and 9.02 ± 2.11 ng/g w.w. in testes of male fish exposed to 0, 0.6, 3 or 15 μg Hg²⁺/L, respectively. Significantly greater concentrations of tHg were observed in ovaries and testes of fish exposed to 15 μg Hg²⁺/L, compared to the control not exposed to Hg (Fig. 2A).

In the F1 generation, concentrations of tHg were 5.50 ± 0.65, 5.99 ± 0.53, 6.34 ± 1.11 and 11.14 ± 1.02 ng/g w.w. in larvae derived from fish exposed to 0, 0.6, 3 or 15 μg Hg²⁺/L, respectively. Concentrations of tHg were 6.48 ± 1.29, 7.88 ± 1.65, 7.39 ± 2.03 and 8.72 ± 1.57 ng/g w.w. in ovaries of female fish, and were 5.32 ± 0.24, 5.73 ± 0.55, 5.96 ± 0.78 and 6.14 ± 0.53 ng/g w.w. in testes of male fish from 0, 0.6, 3 and 15 μg Hg²⁺/L groups, respectively. Exposure to 15 μg Hg²⁺/L led to significantly greater concentrations of tHg in whole larvae, but not in gonads, of the F1 generation (Fig. 2B).

Fig. 4. Histology of ovaries (A-D) and testes (E-H) in F0 generation of fish exposed for 5 days during early life stages to various concentrations of Hg²⁺. (A and E), control; (B and F), 0.6 μg Hg²⁺/L; (C and G), 3 μg Hg²⁺/L; (D and H), 15 μg Hg²⁺/L. PG: primary growth follicles; PV: previtellogenic follicles; EV: early vitellogenic follicles; MV: midvitellogenic follicles; FG: full grown follicles; SG: spermatogonia; SC: spermatocyte; ST: spermatids; SZ: spermatozoa. Scale bars are 500 μm.
In the F$_2$ generation, concentrations of tHg were 5.52 ± 0.57, 5.70 ± 0.62, 5.85 ± 0.76 and 5.71 ± 0.76 ng/g w.w. in larvae from 0, 0.6, 3 and 15 μg Hg$^{2+}$/L groups, respectively. Concentrations of tHg were 5.51 ± 0.45, 5.76 ± 0.58, 6.03 ± 0.57, 5.99 ± 0.46 ng/g w.w. in ovaries of female fish, and were 5.50 ± 0.37, 5.56 ± 0.51, 5.60 ± 0.45, 5.52 ± 0.49 ng/g w.w. in testes of male fish from 0, 0.6, 3 and 15 μg Hg$^{2+}$/L groups, respectively. There were no significant differences in concentrations of tHg in larvae or gonads among treatments, including controls (Fig. 2C).

3.2. GSI and fecundity

In the F$_2$ generation, concentrations of tHg were 5.52 ± 0.57, 5.70 ± 0.62, 5.85 ± 0.76 and 5.71 ± 0.76 ng/g w.w. in larvae from 0, 0.6, 3 and 15 μg Hg$^{2+}$/L groups, respectively. Concentrations of tHg were 5.51 ± 0.45, 5.76 ± 0.58, 6.03 ± 0.57, 5.99 ± 0.46 ng/g w.w. in ovaries of female fish, and were 5.50 ± 0.37, 5.56 ± 0.51, 5.60 ± 0.45, 5.52 ± 0.49 ng/g w.w. in testes of male fish from 0, 0.6, 3 and 15 μg Hg$^{2+}$/L groups, respectively. There were no significant differences in concentrations of tHg in larvae or gonads among treatments, including controls (Fig. 2C).

3.2. GSI and fecundity

In the F$_0$ generation, significantly lesser GSI was observed in both females and males exposed to 15 μg Hg$^{2+}$/L compared to the control group (Fig. 3A). However, this was not observed in F$_1$ or F$_2$ generations (Fig. 3B and C).

Results of the 21-d reproduction study showed that exposure to 3 or 15 μg Hg$^{2+}$/L during early life stages caused lesser cumulative production of eggs in the F$_0$ generation, relative to that of controls (Fig. 3D). However, no obvious effects of treatments to the F$_0$ generation were observed in cumulative production of eggs by F$_1$ (Fig. 3E) or F$_2$ generations (Fig. 3F).

3.3. Gonadal histology, oogenesis and spermatogenesis

For females of the F$_0$ generation, ovaries of controls were filled with follicles at various developmental stages, including PG, PV, EV, MV and FG follicles (Fig. 4A). Compared with the control, no obvious changes in ovarian histology occurred in females exposed to 0.6 μg Hg$^{2+}$/L
However, a greater percentage of early-stage follicles and a reduced percentage of late-stage follicles were observed in fish exposed to 3 or 15 μg Hg²⁺/L (Fig. 4C and D), which was further supported by statistical results of the percentage of follicles at different stages (Supplementary Fig. S1A).

For males of the F₀ generation, differentiated spermatogenic lobules containing germ cells at various developmental phases, including spermatogonia, spermatocytes, spermatids and spermatozoa were present in control testes (Fig. 4E). Compared with the controls, no evident changes of testicular structure were detected in fish exposed to 0.6 or 3 μg Hg²⁺/L (Fig. 4F and G). However, retarded spermatogenesis with increased early-stages germ cells including spermatogonia and spermatocytes, and fewer late-stage germ cells (spermatozoa) were observed in testes of fish exposed to 15 μg Hg²⁺/L (Fig. 4H). Male fish exposed to 15 μg Hg²⁺/L exhibited fewer sperm cells than what was observed in controls (Supplementary Fig. S1B). Exposure to 15 μg Hg²⁺/L down-regulated expressions of mRNA of genes involved in cell proliferation (2.5-, 3.6- and 2.2-fold for pcna, cyclinb1 and cyclind1, respectively) and meiosis (1.7-fold for sycp3 and 2.6-fold for dmc1) during spermatogenesis (Supplementary Fig. S2).

In the F₁ generation, the percentage of follicles in the PG stage was greater, while the proportion at the FG stage was less in females exposed to 15 μg Hg²⁺/L (Supplementary Fig. S1C; Supplementary Fig. S3). No significant alteration in numbers of sperm (Supplementary Fig. S1D) and histology of the testes (Supplementary Fig. S3) were observed in males.

In the F₂ generation, histology of ovaries and testes, the percentage of follicles at different stages, and the number of sperm were not notably different among exposures, including the controls (Supplementary Fig. S1E and F; Supplementary Fig. S4).

3.4. Concentrations of sex hormones

In the F₀ generation, exposure to Hg²⁺ during early life stages did not significantly affect concentrations of 11-KT in either sex relative to controls (Fig. 5). However, concentrations of E2 and the E2/11-KT ratio were lesser in females exposed to 3 or 15 μg Hg²⁺/L (Fig. 5A), but were significantly greater in males exposed to 15 μg Hg²⁺/L (Fig. 5B). These changes were not observed in F₁ and F₂ generations, except for significantly lesser concentrations of E2 in F₁ females (Fig. 5C, D, E and F).

3.5. mRNA of genes in the HPG-axis

In F₀ adult females, exposure to 3 or 15 μg Hg²⁺/L during early life stages down-regulated expression of mRNA of gnrh3 (2.1- and 2.9-fold, respectively), cyp19a1a (1.6- and 1.7-fold, respectively) and erα (1.7- and 1.9-fold, respectively) (Fig. 6A). Exposure to 15 μg Hg²⁺/L down-regulated expression of mRNA of lhb (2.3-fold), cyp19a1b (2.3-fold) and lhr (2.0-fold). In F₀ adult males (Fig. 6B), exposure to 3 or 15 μg Hg²⁺/L during early life stages up-regulated expression of mRNA of cyp19a1b (2.8- and 2.9-fold, respectively) and erα (1.4- and 1.6-fold, respectively).
2.4-fold, respectively. Exposure to 15 μg Hg^{2+}/L during early life stages down-regulated expression of mRNA of cyp19a1b (1.5-fold) in brain as well as lhr (5.3-fold) and cyp19a1a (6.7-fold) in ovaries, but there were no significant effects on expression of mRNA of other genes (Fig. 6C). In F1 adult males (Fig. 6D), there were no significant effects of Hg^{2+} on any tested genes among exposures. In F2 adult fish (Fig. 6E and F), there were no differences in expressions of mRNA of any tested genes in either females or males exposed to any concentrations of Hg^{2+}.

4. Discussion

4.1. Exposure to Hg^{2+} early in life impaired subsequent reproduction in adults

4.1.1. Exposure to Hg^{2+} early in life resulted in accumulation of Hg, delayed gonadal development and lesser fecundity

Fish can absorb and accumulate Hg from food and surrounding water, and thus greater concentrations of Hg in aquatic environments results in accumulation of Hg in tissues of fishes (Monteiro et al., 2013). In embryonic and larval stages, fish can rapidly absorb Hg from surrounding water and greatest amounts of Hg are accumulated in the olfactory epithelium and kidney (Devlin, 2006; Korbas et al., 2011). In adult fish, although, due to their key roles in metabolic regulation and detoxification, the brain, kidney and liver are the main target organs for accumulation of Hg, accumulation in gonads has also been observed (Zhang et al., 2016b). Exposure of zebrafish to 15 μg Hg^{2+}/L during early life stages resulted in significantly greater concentrations of Hg in ovaries and testes of F0 adults. This might be attributed to gradual transfer of Hg absorbed by fish to other tissues via blood circulation (Morcillo et al., 2017).

Results of this work are consistent with those of other studies where exposure of fish to Hg has been observed to damage reproductive function, which in turn leads to a lesser fecundity. For example, no spawning occurred at and above 1.02 μg/L in fathead minnows (Pimephales promelas) after 41 weeks of exposure to Hg (Snarski and Olson, 1982). Spawning success was inversely proportional to concentrations of Hg in bodies of fathead minnows exposed to Hg through the diet of juveniles through to sexual maturation (Drevnick and Sandheinrich, 2003).

Normal development of gonads is essential to guarantee reproductive function of fish. GSI is often used as not only a quantitative index to evaluate maturity of fish, but also is a vital indicator to assess toxic effects of contaminants on gonads (Liu et al., 2016). Lesser GSI in both males and females in Hg-exposed groups indicated inhibited testicular and ovarian growth and delayed gonadal development (Li et al., 2019). Though macroscopic measurements based on gonad size or GSI can describe general development of gonads, histological examination provides a more accurate and effective assessment of stages of development of gonad (Prusina et al., 2014). Consistent with changes in GSI, retarded development of gonads, characterized by increased early-stages follicles/germ cells and decreased late-stages follicles/germ cells, was observed after exposure of early stages to Hg^{2+} (especially in 15 μg Hg^{2+}/L groups), which might be the primary cause for fewer production of eggs in fish exposed to Hg.

4.1.2. Exposure to Hg^{2+} early in life disturbed sex hormone homeostasis and gametogenesis

Homeostasis of sex hormones plays a crucial role in development of gonads and gametogenesis (Nagahama and Yamashita, 2008; Haté et al., 2012). Moreover, the E2/11-KT ratio can be considered as an indicator of abnormal status of sex hormones in fish (Jo et al., 2014). In this work, lesser concentrations of E2 in blood plasma, along with a lesser E2/11-KT ratio were detected in females exposed to Hg^{2+} during early life stages, which suggested that Hg could affect development of follicles through interfering with synthesis of E2. Lesser concentrations of E2 were reported in fathead minnows exposed to methylmercury in their diet (Drevnick and Sandheinrich, 2003), which might have been responsible for delayed gonadal development during Hg-intoxicated. In males, although exposure to Hg^{2+} during early life stages had no effect on concentrations of 11-KT in blood plasma, greater concentrations of E2 with a greater E2/11-KT ratio was observed, which could, in part, account for fewer spermatozoa observed in testes (Wang et al., 2016). Greater concentrations of E2 in blood plasma of males were probably a consequence of up-regulation of cyp19a1b encoding aromatase which transforms testosterone (T) to E2. To further understand the mechanism by which early exposure to Hg^{2+} caused a decrease in sperm count in males, expression of mRNA of key regulatory factors involved in spermatogenesis were examined. Both genes involved in cell proliferation (pcmt, cyclinb1 and cyclinD1) and meiosis (sycp3 and dmc1) during spermatogenesis were down-regulated in fish exposed to Hg, a result that was consistent with the decreased sperm amount. Taken together, exposure of early life stages to Hg^{2+} disturbed the balance of sex hormones and gametogenesis, which resulted in delayed development of gonads and lesser GSI, which in turn resulted in lesser fertility.

4.1.3. Exposure to Hg^{2+} early in life altered expression of mRNA for key genes involved in the HPG-axis

The HPG-axis, a crucial reproductive endocrine control mechanism, directly regulates sex hormone homeostasis and maintains normal reproductive function (Maruska and Fernald, 2011). Gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus regulates synthesis and release of gonadotropins from the pituitary (i.e. follicle-stimulating hormone (FSH) and luteinizing hormone (LH)), which makes it a key mediator of reproduction in fish (Yaron and Levavi-Sivan, 2011). FSH and LH are transported to gonads through circulation of blood and bind specifically to their receptor (FSHR and LHR), triggering production of sex steroids, gametogenesis and gonadal maturation, and ovulation in females and release of sperm in males (Breton et al., 1998; Weltzien et al., 2004). Therefore, disruption of GnRH and secretion of gonadotropins can adversely affect fecundity. Assessment of changes in expressions of mRNA for these genes producing these substances in the HPG-axis can potentially provide information on mechanisms by which Hg can impair reproductive (Crump and Trudeau, 2009). Results of the study reported here showed that expression of mRNA for the genes gnrh3 and lhβ in brain and lhr in gonads were down-regulated in both sexes by exposure to Hg^{2+} during early life stages. Down-regulation of expression of mRNA for fshβ in brains and fshr in testes was also observed in adult males exposed to Hg during early life stages. These results suggested that Hg could inhibit synthesis and secretion of GnRH and gonadotropins via suppressing their transcripts, which subsequently resulted in abnormal production of sex hormones and delayed development of gonads. Similarly, down-regulation of mRNA for gnrh3, lhβ and lhr was observed previously where adult zebrafish were exposed to Hg^{2+} (Zhang et al., 2016b). While expression of mRNA for both fshβ and lhβ were down-regulated by exposure of males to Hg early in life, only expression of mRNA for lhβ was down-regulated in females. Expression of mRNA for lhβ in females remained stable, which in agreement with results of a previous study (Zhang et al., 2016b) and could be attributed to the greater response of mRNA for lhβ to GnRH implantation than that of fshβ in maturing fish (Yaron and Levavi-Sivan, 2011).

In fishes, cytochrome P450 aromatase (CYP19) is a key enzyme in steroidogenesis, which catalyzes androgens (e.g. T) into estrogens (e.g. E2) (Trant et al., 2001). Two different cyp19 genes encoding aromatase
have been reported in zebrafish, referred to as *cyp19a1b* and *cyp19a1a*, mainly expressed in brain and ovary, respectively (Chiang et al., 2001; Kishida and Callard, 2001). The increase or decrease in production of E2 is closely related to alteration in expression of mRNA for the genes as well as activity of the enzyme CYP19 (Su et al., 2016; Xu et al., 2017). In this study, down-regulation of expression of mRNA for *cyp19a1b* in brains and *cyp19a1a* and *erα* in ovaries were observed in adult, female fish exposed to Hg during early life stages. This result corresponded with lesser concentrations of E2 in blood plasma. In males, up-regulation of *cyp19a1b* in brains and *erα* in testes also paralleled greater concentrations of E2 in blood plasma. These results indicated that exposure to Hg altered the concentration of E2 by modulating transcription of genes encoding for the enzyme aromatase, thereby interfering with the balance among steroid sex hormones in adult fish. Gonadotropins can also regulate production of steroid sex hormones in gonads. Although expressions of mRNA of the genes *gnrh3*, *fshβ*, *lhβ*, *fshr* and *lhr* upstream of the HPG-axis were down-regulated, a greater concentration of E2 in blood plasma of adult males was still observed, which suggested a negative feedback regulation mechanism in the HPG-axis had been activated to compensate for greater concentration of E2 by down-regulating expression of mRNA of genes upstream of the HPG-axis (Xu et al., 2017). In addition, CYP11B is responsible for conversion of T to 11-KT (Sower et al., 2009). No significant changes in concentrations of 11-KT or mRNA coding for *ar*, which was observed in males that had been exposed to Hg during early life stages could be related to the fact that no alteration in expression of mRNA of *cyp11b2* was observed in this study. Therefore, it can be postulated that Hg disrupted homeostasis of steroid sex hormones, which resulted in delayed development of gonads by altering expression of mRNA of key genes along the HPG-axis.

4.2. Intergenerational effects and recovery reproductive impairment of adults after exposure of early life stages to Hg

Although results of multiple studies have demonstrated toxic effects of Hg on fishes, possible intergenerational effects of its toxicity remained poorly understood. Maternal transfer is one of the major routes for...
embryos or larvae of fish to be exposed to metals (Devlin, 2006; Morcillo et al., 2017). In the case of significant concentrations of Hg in oocytes and testes of F₀ fish, it was not surprising that concentrations of Hg in larvae of F₁ generation increased significantly. This result indicated that Hg was transferred to offspring, which was consistent with results of other studies (Bridges et al., 2016; Mora-Zamorano et al., 2016). In gonads of the F₁ generation, there were no evident differences in concentrations of Hg among treatments although an increasing trend was observed, which indicated that Hg absorbed by fish was gradually diluted and eliminated.

Similar to those observed in females of the F₀ generation, exposure of early life stages to Hg²⁺ resulted in delayed development of oocytes and lesser concentrations of E₂ in F₁ females. This response could be related to down-regulation of expression of mRNA of cyp19a1b in brains and lhr and cyp19a1a in oocytes. Since offspring were not directly exposed to Hg, it was likely intergenerational effects of Hg on reproduction. Therefore, zebrafish can be considered as a model for understanding Hg-induced intergenerational toxicity, as reported in other toxic substances (Wu et al., 2017; Hedayatirad et al., 2020). There was no obvious difference in GSI or cumulative production of eggs in F₁ females, although a trend of lesser production was observed. Meanwhile, no significant changes were observed in any of the measured indicators, including accumulation of Hg, GSI, fecundity, histology, concentrations of sex hormones or, expression of mRNA of genes involved in the HPG-axis of F₁ males. These results demonstrated that reproductive damage caused by parental early life exposure to Hg²⁺ was partially recovered in F₁ females of the F₀ generation, but fully recovered in males of the F₁ generation. Further studies are needed to investigate the mechanisms by which females were more persistently affected by exposure to Hg²⁺ during early life stages than males.

In order to further determine whether impaired reproduction observed in adult females exposed to Hg²⁺ during early life stages could fully recover and whether the males had fully recovered, indicators mentioned above were also measured for the F₂ generation. These indicators returned to the values of the controls, which could be attributed to the dispersion of Hg in fish because no significant increase in concentrations of Hg was detected in either larvae or gonads of the F₂ generation. Taken together, early life exposure to Hg²⁺ resulted in intergenerational damage to the reproductive system in un-exposed F₁ females, but this damage was not observed in males of the F₁ generation or in either sex of the F₂ generation.

5. Conclusions

Exposure of zebrafish during early life stages to environmentally relevant concentrations of Hg²⁺ disturbed the balance of sex hormones and gametogenesis by altering expressions of mRNA for key genes in the HPG-axis, and subsequently causing delay in development of the gonad and lesser GSI. Together these effects, resulted in lesser fecundity. A similar, but less pronounced effect, was observed in un-exposed F₁ females, which suggested intergenerational impairment of Hg²⁺ on reproduction of female fish. However, this damage was not observed in males of the F₁ generation or in either sex of the F₂ generation, which indicated that reproductive damage caused by exposure to Hg²⁺ during early life stages were recovered (Fig. 7). Considering exposure to Hg²⁺ early in life-history can adversely affect the reproduction of fishes, the potential impacts of inorganic Hg on the population dynamics of aquatic animals should deserve more attention. Moreover, it is also important to concern whether organisms have ever been exposed to Hg.

CRediT authorship contribution statement


Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

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References


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