Chemosphere xxx (xxxx) 128768

Contents lists available at ScienceDirect

Chemosphere

journal homepage: http://ees.elsevier.com

Effects of 3,4-dichloroaniline (3,4-DCA) and 4,4′-methylenedianiline (4,4′-MDA) on sex hormone regulation and reproduction of adult zebrafish (*Danio rerio*)

Md Nurul Huda Bhuiyan [a,b], Habyeong Kang [a], Jiwon Choi [a], Soyoung Lim [a], Younglim Kho [c], Kyungho Choi [a,*]

[a] Graduate School of Public Health, Seoul National University, Seoul, 08826, Republic of Korea
[b] Bangladesh Council of Scientific and Industrial Research, Dhaka, 1205, Bangladesh
[c] Department of Health, Environment and Safety, Eulji University, Seongnam, 34824, Republic of Korea

ARTICLE INFO

Article history:
Received 11 July 2020
Received in revised form 16 October 2020
Accepted 26 October 2020
Available online xxx
Handling Editor: James Lazorchak

Keywords
3,4-dichloroaniline
4,4′-methylenedianiline
Reproduction
Sex hormones
Endocrine
Zebrafish

ABSTRACT

3,4-dichloroaniline (3,4-DCA) and 4,4′-methylenedianiline (4,4′-MDA) have been widely used in manufacture of many industrial and consumer products, and hence often detected in aquatic environment. Reproductive toxicity of aniline and its derivatives in aquatic organisms has been suggested, however, knowledge on the endocrine disruption potentials and toxicological consequences of both anilines are not well understood, especially in fish. In this study, we aimed to understand the effects of 3,4-DCA and 4,4′-MDA on sex hormone regulation and reproduction of adult zebrafish (*Danio rerio*). Following 21 d exposure, significant decreases of the reproduction were observed at 0.38 mg/L 3,4-DCA, and 4.6 mg/L 4,4′-MDA. Moreover, plasma concentrations of testosterone (T) and 17β-estradiol (E2) level were significantly decreased in both male and female fish following the exposure. The sex hormone changes could be explained by the regulatory changes of the genes along the hypothalamic-pituitary-gonadal (HPG) axis, including significant down-regulation of *steroidogenic acute regulatory protein* (*star*) and *cytochrome P450 family 19 subfamily A* (*cyp19a*) genes in the gonad. Moreover, inhibition of gonadotropin hormone signaling and *prostaglandin-endoperoxide synthase 2* (*ptgs2*) gene expression were observed, suggesting potential disruption of oocyte maturation and ovulation by the exposure. Our observations indicate that 3,4-DCA and 4,4′-MDA can impair reproduction of zebrafish potentially through disruption of steroid hormone synthesis and ovulation.

© 2020

1. Introduction

Aniline is an aromatic amine family with hundreds of derivatives. Among them, 3,4-dichloroaniline (3,4-DCA) and 4,4′-methylenedianiline (4,4′-MDA) are two most widely used aniline derivatives, and are classified as high volume production chemicals in several countries such as USA, EU, and Korea (Di Girolamo et al., 2009; European Commission, 2012, 2012; Ministry of Environment, 2007; Sihtmäe et al., 2010; US EPA, 2009). 3,4-DCA has been used in herbicides, dyes, and paints as precursors or intermediates (Mattarozzi et al., 2013; Saleh et al., 2016; Yuan et al., 2017), and 4,4′-MDA in industrial polyurethane, flexible and rigid foams, elastomers, adhesives, binders, coating, and paints (Lewis, 2007; Mattarozzi et al., 2013).

Since 3,4-DCA can easily enter the environment not only during the manufacturing processes but also following the application (European Commission, 2006), this compound has been frequently detected in the environment especially in the water (Boulahlib et al., 2016; Jurado-Sanchez et al., 2012; Saleh et al., 2016; Yao et al., 2011; Zhao et al., 2001). In USA, 3,4-DCA has been detected in the Sope Creek and Chattahoochee River at concentrations up to 68.2 ng/L (US Geological Survey, 2012). As a building block of many chemicals, the parent compounds of anilines that are used as herbicides and drugs are also detected in the aquatic environment (Balakrishnan et al., 2012; Sapozhnikova et al., 2013). For example, diuron, a parent compound of 3,4-DCA, was detected in the harbor area at up to 230 ng/L, and up to 1360 ng/L in the fishing port area of Jinhae Bay, Korea (Kim et al., 2014).

While environmental occurrences have seldom been reported, 4,4′-MDA can also enter the environment through industrial effluents or through leaching from the consumer materials such as rubber products and adhesives during the use. In addition, 4,4′-MDA can be generated from the residual isocyanates remaining in polyurethane adhesive (Campanella et al., 2015; Pezo et al., 2012; Rubio et al., 2014). Once released into the environment, 4,4′-MDA can bind covalently with the organic matters in the soil and sediment, and therefore is not readily degradable in the environment (ATSDR, 1998). This aniline derivative is included in the human biomonitoring for European Union

* Corresponding author. Department of Environmental Health Sciences, Republic of Korea.
E-mail address: kyungho@snu.ac.kr (K. Choi)

https://doi.org/10.1016/j.chemosphere.2020.128768
0045-6535/© 2020.

UNCORRECTED PROOF

(HBM4EU) as one of priority chemicals for biomonitoring (HBM4EU, 2017).

3,4-DCA was reported to affect the development and reproduction of aquatic organisms, and its toxicity was greater than that of its parent herbicide, i.e., diuron (Bozena and Danuta, 1998; Palau-Casellas and Hutchinson, 1998; Ramos et al., 2002). In a freshwater cladoceran, *Daphnia magna*, 3,4-DCA influenced reproduction rate and the age of first reproduction, at the concentration as low as 9 μg/L (Trubetskova and Lampert, 2002). In a freshwater rotifer, *Brachionus calycforus*, significant decreases of net reproductive rate, generation time, and reproduction were observed at 2.5 mg/L (Ferrando et al., 1993). 3,4-DCA has also been reported to cause dose-dependent growth retardation and malformation in a larval rare minnow (*Gobiocypris rarus*) (Zhu et al., 2013).

In addition, experimental evidences suggest endocrine disruption potentials and associated consequences of the aniline derivatives (Holm et al., 2015). For example, estrogenic effect of 3,4-DCA, i.e., increased 17β-estradiol (E2) in female and decreased testosterone (T) in male fish, was observed in Nile tilapia (*Oreochromis niloticus*) (Pereira et al., 2015, 2016). Previously, we have reported significant decreases of T and E2 concentrations in adult male zebrafish (*Danio rerio*) along with down-regulation of some steroidogenic genes following 14 d exposure to 3,4-DCA and 4,4′-MDA (Bhuiyan et al., 2019). However, there remains a significant knowledge gap on the detailed mechanism of sex hormone disruption induced by the aniline derivatives, and their effects on reproduction.

The purpose of this study was to investigate the effects of 3,4-DCA and 4,4′-MDA on sex hormone regulation and reproduction in adult zebrafish. Zebrafish has been frequently used for investigating sex hormone disruption and related mechanisms by exposure to environmental chemicals (Ji et al., 2013; Liu et al., 2012; Ma et al., 2012; Segner, 2009; Sohn et al., 2016; Wang et al., 2015). The results of this study will help understand the endocrine disruption potentials induced by the exposure to two most widely used anilines, and attract follow-up investigations on ecological consequences of 3,4-DCA and 4,4′-MDA exposure in aquatic ecosystem.

2. Material and methods

2.1. Chemicals

3,4-DCA (CAS No. 95-76-1, purity: ≥98%) and 4,4′-MDA (CAS No. 101-77-9, purity: ≥97%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). As a solvent, dimethyl sulfoxide (DMSO) was used. The final concentration of DMSO in the exposure media was set at 0.005% (v/v).

2.2. Zebrafish maintenance and exposure

Both male and female adult zebrafish (wild type, 6 months old) were purchased from a commercial vendor (Green Fish, Seoul, Korea). The fish were then acclimated in the laboratory at least for 15 d before being used for the experiment. The water temperature was maintained at 26 ± 1 °C, and the photoperiod was set at 14:10 h light:dark. The exposure concentrations for 3,4-DCA were determined at 0.024, 0.12, and 0.6 mg/L; and for 4,4′-MDA were 0.2, 1.0, and 5.0 mg/L, in addition to water control (C) and solvent control (SC), based on the results of preliminary range-finding experiments. For the range-finding experiments, fish were exposed to each target compound for 7 d at several serially diluted concentrations (three replicates per concentration, five fish per replicate). The exposure media was renewed, and the fish survival was checked daily during the range-finding tests. The concentrations at which less than 10% of fish died were chosen as the experimental concentrations for the definitive tests.

For a fish short-term reproduction assay, OECD test guideline 229 was followed, with minor modifications on the fish sex ratio and water renewal (OECD, 2009). The fish were fed with freshly hatched *Artemia* nauplii twice a day. During the exposure, about 90% of the exposure media were renewed daily with freshly prepared media. The water quality parameters including dissolved oxygen, conductivity, temperature, and pH were monitored between the media renewal.

Before the exposure, the pairs of fish were allowed to mate for 7 d to confirm similar fecundity across the mating pairs. For exposure, eight replicates per treatment or control were prepared, with two male and two female fish in a replicate (Fig. 1). During the exposure duration, the number of embryos produced (reproduction) was counted every morning approximately 30 min after the lighting. At the conclusion of the 21 d exposure, fish were sacrificed, and the samples including blood, brain, and gonad were sampled. The fish blood was collected from the caudal vein using a capillary tube. Because of the limited sample volume, the blood samples from four fish of the same sex, obtained from two replicates, were pooled for one measurement. For each treatment or control, therefore, four pooled blood samples were prepared. The pooled blood was then centrifuged (8000 rpm for 10 min at 4 °C) and the plasma was separated and stored at −80 °C. Brain and gonad samples were also pooled: The samples collected from three fish of the same sex, randomly obtained from two replicates, were pooled (n = 4 per treatment). These pooled organs were stored at −80 °C until analysis for gene transcription. This work was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (SNU-190114-5).

2.3. Chemical analysis in fish exposure media

The test chemicals were measured in the fish exposure media between water renewal, i.e., at the beginning (0 h) and after the 24 h of the exposure (Bhuiyan et al., 2019). Briefly, 1 mL of the media was sampled, and analyzed using an ultra-high pressure liquid chromatography (UHPLC) system (Nexera, Shimadzu Corporation, Kyoto, Japan) coupled with API 4500 Triple Quadrupole Mass Spectrometry System (AB SCIEX, Ontario, Canada). Details of analytical procedures are described in Bhuiyan et al. (2019) The limit of detection was 0.22 ng/mL for 3,4-DCA, and 0.15 ng/mL for 4,4′-MDA. The average concentrations of the 0 h and 24 h water measurements were used for presentation of the results throughout this study (Table S1; Bhuiyan et al., 2019).

2.4. Measurement of sex hormones and related gene expressions

Two sex hormones, i.e., E2 and T, were measured in the blood plasma of male and female zebrafish. Commercial kits employing enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical; E2 [Cat No. 582251] and T [Cat No. 582701]) were used for this purpose. Briefly, hormone extraction was performed using 10 μL of fish plasma. The plasma was diluted to 400 μL with ultrapure water. Then, 2 mL of diethyl ether, an extraction solvent, was added to the diluted sample

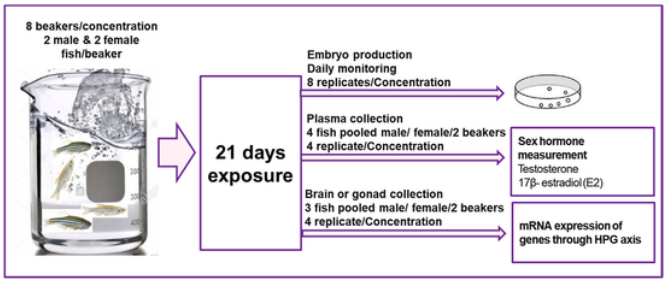

Fig. 1. Experimental design to investigate the effects of 3,4-DCA and 4,4′-MDA on sex hormone regulation and reproduction of adult zebrafish following 21 d exposure.

UNCORRECTED PROOF

and the sample was centrifuged (2100 g, 10 min). After the centrifugation, the upper layer was collected. This extraction procedure was repeated twice. The extraction solvent of the entire samples was evaporated under the nitrogen flow. The dried samples were diluted with 120 μL EIA buffer and used for the hormone measurement (Ji et al., 2010).

For major genes of hypothalamic-pituitary-gonadal (HPG) axis, the tissue samples, i.e., brain and gonad, were homogenized before the total RNA isolation. The extraction of total RNA was carried out using RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacture's instruction. The complementary DNA (cDNA) was synthesized with 100 ng/μL of the extracted RNA, using an iScriptTM cDNA synthesis kit (BioRad, Hercules, CA, USA). The concentration and purity of the extracted RNA and synthesized cDNA were confirmed by measuring absorbance of 260 and 280 nm using Epoch™ microplate reader (BioTek, Winooski, VT, USA). Light Cycler 480 (Roche Applied Science, Indianapolis, IN, USA) was employed for qRT-PCR. As a reference gene, *β-actin* was selected, because this gene was found most stable among three candidate housekeeping genes of *β-actin*, *ribosomal protein L8* (*rpl8*), and *18s ribosomal RNA* (*18srrna*) following the exposure to both anilines in brain and gonads of male and female fish (results not shown). The relative transcription of each target gene was normalized with that of *β-actin*. The expression level was calculated by the threshold cycle (C*t*) number using the ΔΔCt method (Livak and Schmittgen, 2001). The primer sequences of the genes investigated in this study were based on Ji et al. (2013), and this information is shown in Tables S2 and S3.

2.5. Statistical analysis

Shapiro-Wilk's test and Levene's test were used for assessment of normality of data and homogeneity of variances, respectively. Log-transformation was performed on the data which did not follow a normal distribution. To compare the differences between the treatments and the control, one-way analysis of variance (ANOVA) followed by Dunnett's test was conducted. All the statistical analyses were carried out using SPSS 23.0 for Windows (SPSS Inc., Chicago, IL, USA). The $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Effects on reproduction

At the maximum experimental concentration, both 3,4-DCA and 4,4′-MDA showed significant reduction in embryo production per spawning event (Fig. 2A). In addition, the cumulative number of embryos and the number of spawning events significantly decreased at the highest concentration of each chemical (Fig. 2B and C). Between the control and solvent control, the reproduction was not different (data not shown).

3.2. Plasma sex hormones

The levels of both sex hormones were altered after the exposure to 3,4-DCA and 4,4′-MDA, in both male and female zebrafish. In male fish, significant decreases in T and E2 level were observed at 0.38 mg/L of 3,4-DCA, and 0.58 and 4.6 mg/L of 4,4′-MDA (Fig. 3A and B). In addition, a decrease of E2 level was observed at 4.6 mg/L of 4,4′-MDA. In female fish, decrease of both T and E2 was also observed. For 3,4-DCA, the extent of E2 decrease was greater than T in both male and female fish, and therefore E2/T ratios decreased by the exposure. For 4,4′-MDA exposure, T decrease was much greater in the male fish, and therefore higher E2/T ratio was observed (Fig. 3C).

3.3. Regulation of major steroidogenic genes in the HPG axis

Following exposure to both aniline derivatives, significant regulatory changes were observed for major steroidogenic genes in both sexes (Figs. 4 and 5; Tables S4 and S5). The transcriptional changes of major genes observed for each sex and organ following exposure to 3,4-DCA and 4,4′-MDA are summarized in Figs. 4 and 5, respectively.

3.3.1. Brain

A significant up-regulation of *gonadotropin releasing hormone 2* (*gnrh2*) gene was observed in both male and female brain at the highest exposure level of 4,4′-MDA. However, following 3,4-DCA exposure, this gene was up-regulated only in the female brain (Figs. 4 and 5). For *gnrh3* gene, 4,4′-MDA exposure resulted in different pattern of regulatory change by sex, i.e., down-regulation in male, and up-regulation in female (Fig. 5). Following exposure to 3,4-DCA at 0.38 mg/L, *gnrh3* gene was up-regulated in the male fish (Fig. 4).

Following exposure to 3,4-DCA, two pituitary genes (*gnrhr2* and *gnrhr4*) were up-regulated in the male fish, but *gnrhr1* was up-regulated only in the female at the highest exposure level (Fig. 4). Similarly, following exposure to 4,4′-MDA, *gnrhr1* gene was up-regulated, whereas *gnrhr2* was down-regulated in the male (Fig. 5). A significant down-regulation of *follicular stimulating hormone β* (*fshβ*) was observed in the male fish following exposure to both anilines. The *fshβ* gene was also down-regulated in the female following 3,4-DCA exposure. In addition, *luteinizing hormone β* (*lhβ*) gene was down-regulated by 4,4′-MDA exposure. The *cyp19b* gene was down-regulated in both male and female fish upon the exposure to both compounds. A significant down-regulation of *estrogen receptor α* (*erα*) was observed in the female by both the compounds, while *erα*, *er2β,* and *androgen receptor* (*ar*) genes were significantly up-regulated following 4,4′-MDA exposure. The expression of *erα*, *er2β,* and *ar* genes were not affected in the male fish following 3,4-DCA exposure.

3.3.2. Gonad

Significant transcriptional changes were observed for several major genes in the testis and ovary at the highest experimental concentration of each compound (Figs. 4 and 5). After exposure to 3,4-DCA, *fshr* gene was down-regulated only in the ovary while no changes were observed for *lhr* gene in both male or female gonads. Following exposure to 4,4′-MDA, *lhr* gene was down-regulated in both testis and ovary, while *fshr* gene was significantly down-regulated in the testis but up-regulated in the ovary. The *3-hydroxy-3-methylglutaryl-CoA reductase a* (*hmgra*) gene was down-regulated in the testis, but no changes were observed in the ovary. A significant up-regulation was observed for *hmgrb* gene in the ovary by 4,4′-MDA exposure. The *star* and *cyp19a* genes were significantly down-regulated in both male and female gonad after exposure to both compounds. In addition, significant down-regulations of *cytochrome P450* family 11 subfamily A (*cyp11a*) and *17β-hydroxysteroid dehydrogenase* (*17βhsd*) were observed by 3,4-DCA and 4,4′-MDA exposure, respectively. A significant up-regulation of *3βhsd* and *cyp17a* genes were also observed in both male and female gonads following 4,4′-MDA exposure. On the other hand, *ptgs2* gene, which is related to oocyte maturation and ovulation, was significantly down-regulated in both testis and ovary by 4,4′-MDA, and in the testis by 3,4-DCA (Fig. 5).

4. Discussion

Significant decrease in reproduction (Fig. 2), disruption of sex hormone regulation (Fig. 3), and alteration of several HPG genes (Figs. 4 and 5) observed in this study demonstrate sex hormone disrupting potential of 3,4-DCA and 4,4′-MDA in zebrafish. This study, for the first time, reports impaired reproduction of zebrafish by 3,4-DCA or 4,4′-

UNCORRECTED PROOF

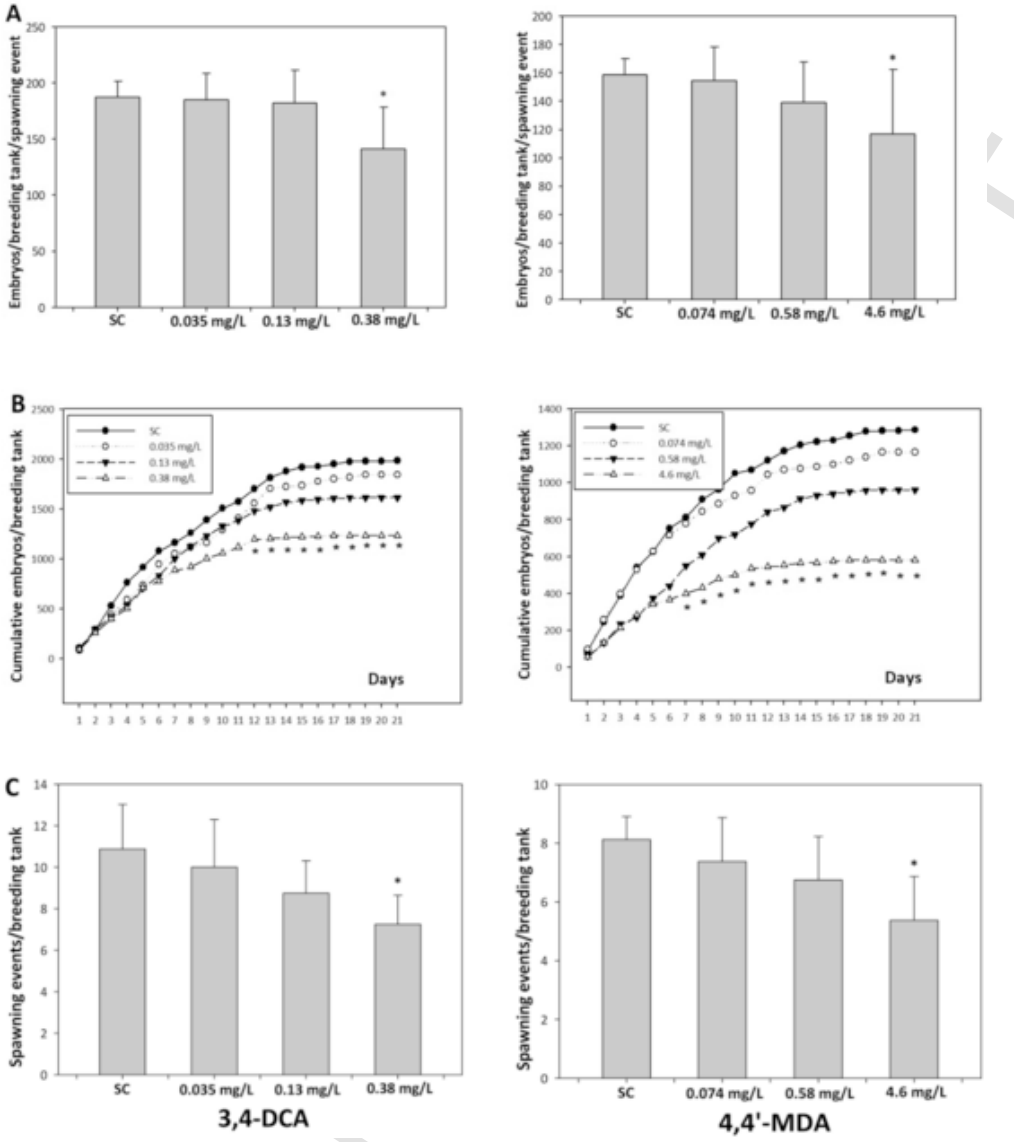

Fig. 2. Effects of 21 d exposure to 3,4-DCA and 4,4′-MDA on the reproduction of zebrafish. A: Embryo production/per breeding tank/spawning; B: Cumulative embryo production/breeding tank); C: Spawning events/breeding tank. One breading tank represents two pairs of zebrafish (n = 8). Asterisk represents a significant difference ($p < 0.05$) from solvent control (SC).

MDA exposure, and suggests sex hormone disruption as potential underlying mechanism. Endocrine disruption effects of 3,4-DCA and 4,4′-MDA were suggested previously (Bhuiyan et al., 2019; Pereira et al., 2015, 2016), but information on the detailed mechanisms of sex hormone disruption and its consequences on reproduction was limited.

Impairment of reproductions in zebrafish by exposure to 3,4-DCA and 4,4'-MDA (Fig. 2) could be supported by alteration of sex hormone balance (Fig. 3). In addition to the changes in E2 and T levels, altered E2/T ratio would provide an information on overall direction of disruption in sex hormone balance: For instance, decreased E2/T ratio can be interpreted as androgenic or anti-estrogenic activity. The E2/T ratio has been used to indicate sex hormone abnormality and linked to impaired fecundity of the fish (Ji et al., 2013; Kwon et al., 2016; Orlando et al., 2004; Shang et al., 2006). Increase of E2/T ratio observed in the male fish exposed to 4,4′-MDA is in line with our previous reports on male zebrafish and human adrenal carcinoma cell (H295R cell line) (Bhuiyan et al., 2019). The opposite direction of E2/T ratio in the female fish implies that endocrine disruption effects of 4,4′- MDA could be sex-dependent. The decrease of E2/T ratio observed in the male fish exposed to 3,4-DCA, which was repeatedly confirmed in another experiment (Fig. S1), requires careful interpretation because this observation is different from our previous observation in the male zebrafish exposed to the same compound for 14 d (Bhuiyan et al., 2019). The discrepant results could be explained by difference in exposure levels or duration between these studies; in Bhuiyan et al. (2019), the significant increase in E2/T ratio was detected only at the highest exposure concentration (1.9 mg/L) while the E2/T ratio was not significantly changed at the lower concentrations (0.035–0.38 mg/L), which are within the same concentration range where the decreasing trend of E2/T ratio was observed in the present study.

Among the genes in HPG axis, transcriptional changes of gonadotropin hormones may provide insight on underlying mechanism of reduced reproduction of the fish. Secretion of gonadotropins is regulated by gonadotropin-releasing hormones (GnRHs) in hypothalamus, and therefore GnRHs play important roles in the reproduction of vertebrates (Okuzawa et al., 2003; Tsutsumi and Webster, 2009). Up-regulations of *gnrh* genes (*gnrh2* and *gnrh3*) along with corresponding *gonadotropin releasing hormone receptors* (*gnrhr1*, *gnrhr2*, and *gnrhr4*) ob-

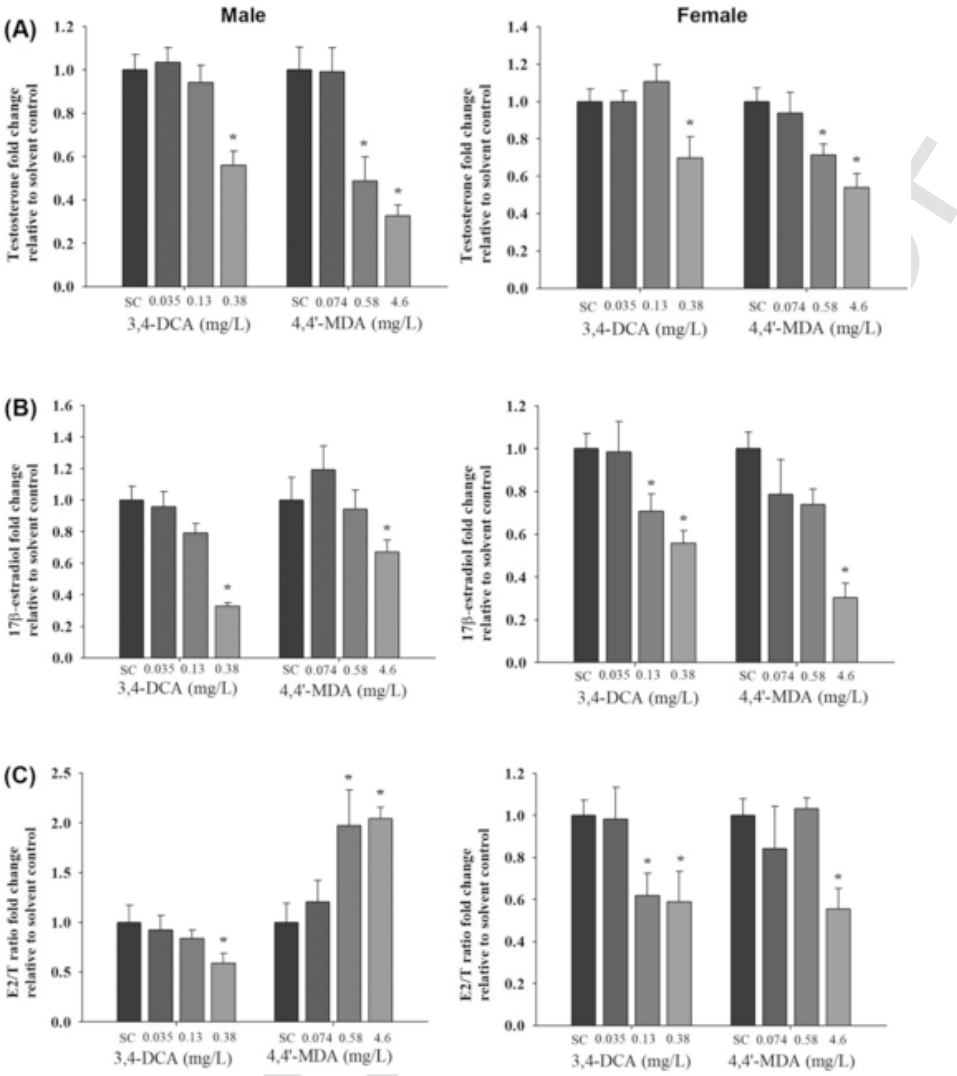

Fig. 3. Changes in (A) testosterone (T), (B) 17β-estradiol (E2) concentrations, and (C) E2/T ratio measured in the plasma of male and female zebrafish following 21 d exposure to 3,4-DCA or 4,4′-MDA. Bar represents the mean and the error bar shows SD of four replicates for each concentration. Asterisk represents a significant difference ($p < 0.05$) from solvent control (SC).

served following 3,4-DCA exposure (Fig. 4) clearly show that a gonadotropin hormone signaling is altered by this compound. Similarly, up-regulation of *gnrh2* in both male and female fish, and sex-dependent regulatory changes of the *gnrh* genes (Fig. 5) suggest that 4,4′-MDA could also alter the release of gonadotropin hormones in the fish.

Two gonadotropin hormones, i.e., fsh and lh, bind to their gonadal receptors, and regulate steroidogenesis and gametogenesis in the fish (Kumar and Trant, 2001; Kwok et al., 2005). The fsh and lh regulate spermatogenesis in male (Ohta et al., 2007; Schulz et al., 2010), and vitellogenesis and oocyte maturation, respectively, in female fish (Clelland and Peng, 2009). In this study, both *fshβ* and *fshr* genes were down-regulated by 4,4′-MDA exposure (Fig. 5). Following 3,4-DCA exposure, *fshβ*, *lhβ*, and *fshr* genes were also down-regulated in the male, while up-regulation of *fshr* and down-regulation of *lhr* gene were observed in the female (Fig. 4). Our observations suggest that the exposure to anilines might influence spermatogenesis and oocyte maturation in the fish, potentially leading to impairment of reproduction.

The sex hormone changes observed in this study is supported by the regulatory alterations of the steroidogenic genes (Figs. 3–5). In the present study, significant decreases of T and E2 concentrations (Fig. 3A and B) were accompanied by the down-regulation of *star* and *cyp19a* genes in both sexes following the exposure to both anilines (Figs. 4 and 5). Steroidogenic acute regulatory protein (star) initiates steroidogenic pathway, by catalyzing a rate-limiting transportation of cholesterol into inner membrane of mitochondria (Muthulakshmi et al., 2018). Therefore, significant down-regulation of *star* may result in decreased cholesterol uptake and eventually lead to reduced syntheses of overall steroid hormones including T and E2. Both down-regulations of *star* gene and reduction of T and E2 levels are in line with a previous report in male zebrafish and H295R following 3,4-DCA and 4,4′-MDA exposure (Bhuiyan et al., 2019).

Aromatase enzyme, cyp19a, is essential for the conversion of T to E2, and hence plays an important role in regulation of E2 level in zebrafish (Fenske and Segner, 2004; Kwon et al., 2016; Uchida et al., 2004). In the present study, down-regulation of *cyp19a* by both anilines (Figs. 4 and 5), therefore explains decreased E2/T ratio observed in most cases except 4,4′-MDA in the male fish (Fig. 3C). While significant increase of E2/T ratio observed in the male fish following 4,4′-MDA exposure warrants further investigation, both sex hormones were similarly decreased in the male fish.

The pattern of transcriptional changes of the other steroidogenic genes, e.g., *hydroxyl methyl glutaryl CoA reductase* genes (*hmgra* and *hm-*

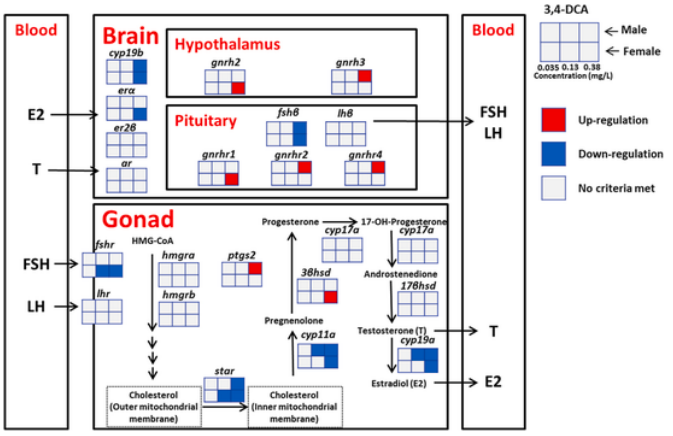

Fig. 4. Effect on the major gene expression related to sex hormone regulation through the HPG axis of zebrafish followingfoll exposure to 3,4-DCA. Each box represents gene expression change of a treatment group from the male or female fish (refer to the legend on the upper right corner of the graph). The direction of gene expression change is indicated by color (Red: significant up-regulation; blue: significant down-regulation; grey: no significant change). Detailed changes of gene transcription can be found in Table S4 of Supporting Information. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

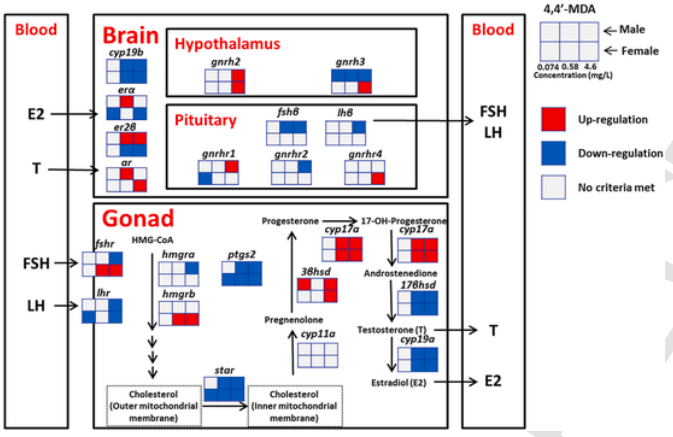

Fig. 5. Effect on the major gene expression related to sex hormone regulation through the HPG axis of zebrafish upon exposure to 4,4′-MDA. Each box represents gene expression change of a treatment group from the male or female fish (refer to the legend on the upper right corner of the graph). The direction of gene expression change is indicated by color (Red: significant up-regulation; blue: significant down-regulation; grey: no significant change). Detailed changes of gene transcription can be found in Table S5 of Supporting Information. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

grb), *cyp11a*, *3βhsd*, *cyp17a*, and *17βhsd*, were different between the anilines, suggesting different modes of steroidogenic alteration. In the fish exposed to 4,4′-MDA, transcriptional changes of *hmgra* and *hmgrb* genes suggest impaired cholesterol supply as this enzyme is responsible for rate-controlling of the mevalonate pathway that produces the cholesterol (Wang et al., 2015). Up-regulation of these steroidogenic genes can be understood as compensation for reduced T and E2 levels, but this observation is not consistent with the results of the 14 d exposure (Bhuiyan et al., 2019). Perhaps longer exposure period in the present study would explain this discrepancy in the gene regulation. Down-regulation of *17βhsd*, which encodes an enzyme responsible for the conversion of androstenedione to T, may support the reduction of T and E2 levels. On the other hand, expressions of those genes were rarely affected following 3,4-DCA exposure, suggesting different response of the fish to these compounds.

In addition, changes of *cyclooxygenase* gene expression after exposure to both 3,4-DCA and 4,4'-MDA, may in part support the present observation of the impaired embryo production (Fig. 2). The cox enzyme catalyzes prostaglandin synthesis in teleost follicles and is also responsible for maturation of oocytes and ovulation (Lister and Van Der Kraak, 2008; Sorbera et al., 2001). The *ptgs2* gene expression may influence reproduction and can be affected by chemical exposure (Garcia-Reyero et al., 2009; Carnevali et al., 2010). Previously, reduced reproduction in zebrafish caused by di-(2-ethylhexyl)-phthalate exposure was associated with expression of genes including *ptgs2*, which are involved in oogenesis. In our study, down-regulation of *cox2* (*ptgs2*) gene was observed in both male and female fish gonads following exposure to 4,4′-MDA, and up-regulation was observed in the male fish by 3,4-DCA exposure.

5. Conclusions

Both 3,4-DCA and 4,4′-MDA could influence sex hormone balance and reproduction performance of zebrafish following 21 d exposure. Altered steroidogenic regulatory pathway induced by exposure to both aniline derivatives may explain hormonal disruption and possibly reproduction damage of zebrafish observed in the present study. Potential disruption of oocyte maturation and ovulation of the fish were also suggested. Further investigations on consequences of longer-term exposure to both anilines and transgenerational effects are warranted.

Author contribution

Md Nurul Huda Bhuiyan: Writing - original draft, Investigation, Formal analysis, Visualization, Habyeong Kang: Investigation, Formal analysis, Jiwon Choi: Investigation, Formal analysis, Soyoung Lim: Investigation, Formal analysis, Younglim Kho: Chemical analysis, Writing - review & editing, Kyungho Choi: Conceptualization, Writing-Reviews & Editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2020R1A2C3011269).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.128768.

Uncited references

; ; ; ; ; ; ; ; ; , .

References

ATSDR, 1998. Agency for toxic substances and disease registry (ATSDR). Toxicological Profile for Methylenedianiline. Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA 1998.

Balakrishnan, S., Takeda, K., Sakugawa, H., 2012. Occurrence of diuron and irgarol in seawater, sediments and planktons of seto inland sea. Japan. Geochem. J 46, 169–177.

Bhuiyan, M.N.H., Kang, H., Kim, J., Kim, S., Kho, Y., Choi, K., 2019. Endocrine disruption by several aniline derivatives and related mechanisms in a human adrenal H295R cell line and adult male zebrafish. Ecotoxicol. Environ. Saf. 180, 326–332.

Boulahlib, S., Boudina, A., Si-Ahmed, K., Bessekhouad, Y., Trari, M., 2016. Development and validation of a fast and simple HPLC method for the

UNCORRECTED PROOF

simultaneous determination of aniline and its degradation products in wastewater. Anal. Methods 8, 5949–5956.
Bozena, S.S., Danuta, T., 1998. Microalgal ecotoxicity test with 3,4-dichloroaniline. Chemosphere 37, 2975–2982.
Campanella, G., Ghaani, M., Quetti, G., Farris, S., 2015. On the origin of primary aromatic amines in food packaging materials. Trends Food Sci. Technol. 46, 137–143.
Carnevali, O., Tosti, L., Speciale, C., Peng, C., Zhu, Y., Maradonna, F., 2010. DEHP impairs zebrafish reproduction by affecting critical factors in oogenesis. PloS One 5, e10201.
Clelland, E., Peng, C., 2009. Endocrine/paracrine control of zebrafish ovarian development. Mol. Cell. Endocrinol. 312, 42–52.
Di Girolamo, F., Campanella, L., Samperi, R., Bachi, A., 2009. Mass spectrometric identification of hemoglobin modifications induced by nitrosobenzene. Ecotoxicol. Environ. Saf. 72, 1601–1608.
European Commission, 2006. 3rd priority list. In: European Union Risk Assessment Report for 3,4-dichloroaniline. Institute for Health and Consumer Products, 65. European chemical bureau EUR 22235EN. https://echa.europa.eu/documents/10162/b41f2253-824c-48f8-b494-df54bd901e6a.
European Commission, 2012. Recommendation from the scientific committee on the occupational exposure limits for 4,4'-Diaminodiphenylmethane (MDA). SCOEL/SUM/107.
Fenske, M., Segner, H., 2004. Aromatase modulation alters gonadal differentiation in developing zebrafish (*Danio rerio*). Aquat. Toxicol. 67, 105–126.
Ferrando, M.D., Janssen, C.R., Audreu, E., Persoone, G., 1993. Ecotoxicological studies with the freshwater rotifer *Brachionus calyciflorus*. II. An assessment of the chronic toxicity of lindane and 3,4-dichloroaniline using life Tables. Hydrobiologia 255 (256), 33–40.
Garcia-Reyero, N., Kroll, K.J., Liu, L., Orlando, E.F., Watanabe, K.H., Sepulveda, M.S., Villeneuve, D.L., Perkins, E.J., Ankley, G.T., Denslow, N.D., 2009. Gene expression responses in male fathead minnows exposed to binary mixtures of estrogen and antiestrogen. BMC Genom. 10, 308.
HBM4EU, 2017. Human Biomonitoring for European Union. HBM4EU priority substances. https://www.hbm4eu.eu/the-substances/aniline-family/. (Accessed 29 March 2019).
Holm, J.B., Chalmey, C., Modick, H., Jensen, L.S., Dierkes, G., Weiss, T., Jensen, B.A., Norregard, M.M., Borkowski, K., Styrishave, B., Martin Koch, H., Mazaud-Guittot, S., Jegou, B., Kristiansen, K., Kristensen, D.M., 2015. Aniline is rapidly converted into paracetamol impairing male reproductive development. Toxicol. Sci. 148, 288–298.
Ji, K., Choi, K., Lee, S., Park, S., Khim, J.S., Jo, Choi, E.H., Zhang, K.X., Giesy, J.P., 2010. Effects of sulfathiazole, oxytetracycline and chlortetracycline on steroidogenesis in the human adrenocarcinoma (H295R) cell line and freshwater fish *Oryzias latipes*. J. Hazard Mater. 182 (1), 494–502.
Ji, K., Liu, X., Lee, S., Kang, S., Kho, Y., Giesy, J.P., Choi, K., 2013. Effects of non-steroidal anti-inflammatory drugs on hormones and genes of the hypothalamic-pituitary-gonad axis, and reproduction of zebrafish. J. Hazard Mater. 254–255, 242–251.
Jurado-Sanchez, B., Ballesteros, E., Gallego, M., 2012. Occurrence of aromatic amines and N-nitrosamines in the different steps of a drinking water treatment plant. Water Res. 46, 4543–4555.
Kim, N.S., Shim, W.J., Yim, U.H., Hong, S.H., Ha, S.Y., Han, G.M., Shin, K.H., 2014. Assessment of TBT and organic booster biocide contamination in seawater from coastal areas of South Korea. Mar. Pollut. Bull. 78, 201–208.
Kumar, R.S., Trant, J.M., 2001. Piscine glycoprotein hormone (gonadotropin and thyrotropin) receptors: a review of recent developments. Comp. Biochem. Physiol. 129B, 347–355.
Kwok, H.F., So, W.K., Wang, Y., Ge, W., 2005. Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle stimulating hormone and luteinizing hormone receptors evidence for their distinct functions in follicle development. Biol. Reprod. 72, 1370–1381.
Kwon, B., Shin, H., Moon, H.B., Ji, K., Kim, K.T., 2016. Effects of tris(2-butoxyethyl) phosphate exposure on endocrine systems and reproduction of zebrafish (*Danio rerio*). Environ. Pollut. 214, 568–574.
Lewis, R.J. Sr, 2007. Hawley's Condensed Chemical Dictionary. fifteenth ed. John Wiley and Sons, Inc., New York, NY, p. 385.
Lister, A.L., Van Der Kraak, G., 2008. An investigation into the role of prostaglandins in zebrafish oocyte maturation and ovulation. Gen. Comp. Endocrinol. 159, 46–57.
Liu, X., Ji, K., Choi, K., 2012. Endocrine disruption potentials of organophosphate flame retardants and related mechanisms in H295R and MVLN cell lines and in zebrafish. Aquat. Toxicol. 114–115, 173–181.
Livak, J.K., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}$CT method. Methods 25, 402–408.
Ma, Y., Han, J., Guo, Y., Lam, P.K., Wu, R.S., Giesy, J.P., Zhang, X., Zhou, B., 2012. Disruption of endocrine function in *in vitro* H295R cell-based and in in vivo assay in zebrafish by 2,4-dichlorophenol. Aquat. Toxicol. 106–107, 173–181.
Mattarozzi, M., Lambertini, F., Suman, M., Careri, M., 2013. Liquid chromatography-full scan-high resolution mass spectrometry-based method towards the comprehensive analysis of migration of primary aromatic amines from food packaging. J. Chromatogr., A 1320, 96–102.
Ministry of Environment, 2007. The 3rd Report 2007 : Investigation on Chemical Amount of Distribution in Korea.
Muthulakshmi, S., Hamideh, F., Habibi, H.R., Maharajan, K., Kadirvelu, K., Mudili, V., 2018. Mycotoxin zearalenone induced gonadal impairment and altered gene expression in the hypothalamic–pituitary–gonadal axis of adult fe1male zebrafish (*Danio rerio*). J. Appl. Toxicol. 38 (11), 388–1397.
OECD, 2009. Fish Short Term Reproduction Assay, OECD Guideline 229, Paris, France. Organisation for Economic Cooperation and Development.
Ohta, T., Miyake, H., Miura, C., Kamei, H., Aida, K., Miura, T., 2007. Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japaneseeel, Anguilla japonica. Biol. Reprod. 77, 970–977.
Okuzawa, K., Gen, K., Bruysters, M., Bogerd, J., Gothif, Y., Kagawa, H., 2003. Seasonal variation of the three native Gonadotropin-releasing hormone messenger ribonucleic acids concentrations in the brain of female red seabream. Gen. Comp. Endocrinol. 130, 324–332.
Orlando, E.F., Kolok, A.S., Binzcik, G.A., Gates, J.L., Horton, M.K., Lambright, C.S., Gray, L.E., Soto, A.M., Guillete, J., 2004. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. Environ. Health Perspect. 112, 353–358.
Palau Casellas, A., Hutchinson, T.H., 1998. Acute toxicity of chlorinated organic chemicals to the embryos and larvae of the marine worm Platynereis dumerilii (Polychaeta: nereidae). Environ. Toxicol. Water Qual. 13, 149–155.
Pereira, T.S., Boscolo, C.N., Felicio, A.A., Batlouni, S.R., Schlenk, D., de Almeida, E.A., 2016. Estrogenic activities of diuron metabolites in female Nile tilapia (*Oreochromis niloticus*). Chemosphere 146, 497–502.
Pereira, T.S., Boscolo, C.N., Silva, D.G., Batlouni, S.R., Schlenk, D., Almeida, E.A., 2015. Anti-androgenic activities of diuron and its metabolites in male Nile tilapia (*Oreochromis niloticus*). Aquat. Toxicol. 164, 10–15.
Pezo, D., Fedeli, M., Bosetti, O., Nerin, C., 2012. Aromatic amines from polyurethane adhesives in food packaging: the challenge of identification and pattern recognition using Quadrupole-Time of Flight-Mass Spectrometry. Anal. Chim. Acta 756, 49–59.
Ramos, E.U., Vaal, M.A., Hermens, J.L.M., 2002. Interspecies sensitivity in the aquatic toxicity of aromatic amines. Environ. Toxicol. Pharmacol. 11, 149–158.
Rubio, L., Sanllorente, S., Sarabia, L.A., Ortiz, M.C., 2014. Optimization of a headspace solid-phase microextraction and gas chromatography/mass spectrometry procedure for the determination of aromatic amines in water and in polyamide spoons. Chemometr. Intell. Lab. Syst. 133, 121–135.
Saleh, A., Molaei, S., Sheijooni Fumani, N., Abedi, E., 2016. Antifouling paint booster biocides (Irgarol 1051 and diuron) in marinas and ports of Bushehr, Persian Gulf. Mar. Pollut. Bull. 105, 367–372.
Sapozhnikova, Y., Wirth, E., Schiff, K., Fulton, M., 2013. Antifouling biocides in water and sediments from California marinas. Mar. Pollut. Bull. 69, 189–194.
Schulz, R.W., Franca, L.R., Lareyre, J., LeGac, Chiarini-Garcia, F.H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.
Segner, H., 2009. Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. Comp. Biochem. Physiol., C 149, 187–195.
Shang, E.H.H., Yu, R.M.K., Wu, R.S.S., 2006. Hypoxia affects sex differentiation and devel-opment, leading to a male-dominated population in zebrafish (*Danio rerio*). Environ. Sci. Technol. 40, 3118–3122.
Sihtmäe, M., Mortimer, M., Kahru, A., Blinova, I., 2010. Toxicity of five anilines to crustaceans, protozoa and bacteria. J. Serb. Chem. Soc. 75, 1291–1302.
Sohn, J., Kim, S., Koschorreck, J., Kho, Y., Choi, K., 2016. Alteration of sex hormone levels and steroidogenic pathway by several low molecular weight phthalates and their metabolites in male zebrafish (*Danio rerio*) and/or human adrenal cell (H295R) line. J. Hazard Mater. 320, 45–54.
Sorbera, L.A., Asturiano, J.F., Carrillo, M., Zanuy, S., 2001. Effects of polyunsaturated fatty acids and prostaglandins on oocyte maturation in a marine teleost, the European sea bass (*Dicentrarchus labrax*). Biol. Reprod. 64, 382–389.
Trubetskova, I., Lampert, W., 2002. The juvenile growth rate of Daphnia: a short term alternative to measuring the per capita rate of increase in ecotoxicology? Arch. Environ. Contam. Toxicol. 42, 193–198.
Tsutsumi, R., Webster, N.J.G., 2009. GnRH pulsatility, the pituitary response and reproductive dysfunction. Endocr. J. 56 (6), 729–737.
Uchida, M., Yamashita, T., Kitano, T., Iguchi, T., 2004. An aromatase inhibitor or high-water temperature induce oocyte apoptosis and depletion of P450 aromatase activity in the gonads of genetic female zebrafish during sex-reversal. Comp. Biochem. Physiol. 137A, 11–20.
US EPA, 2009. Non-confidential production volume information submitted by companies for chemicals under the 1986-2002 inventory update rule (IUR). Benzenamine, 4,4'-methylene bis- (101-77-9) Available from:, as

UNCORRECTED PROOF

of February 18, 2009. http://www.epa.gov/oppt/iur/tools/data/2002-vol.html. (Accessed 20 January 2019).
US Geological Survey, 2012. Analysis of the Herbicide Diuron, Three Diuron Degradates, and Six Neonicotinoid Insecticides in Water—Method Details and Application to Two Georgia Streams. Scientific Investigations Report 2012–5206. US Department of the Interior Available from:. http://pubs.usgs.gov/sir/2012/5206/pdf. (Accessed 20 January 2020).
Wang, J., Cao, X., Sun, J., Huang, Y., Tang, X., 2015. Disruption of endocrine function in H295R cell in vitro and in zebrafish in vivo by naphthenic acids. J. Hazard Mater. 299, 1–9.
Yao, X.F., Khan, F., Pandey, R., Pandey, J., Mourant, R.G., Jain, R.K., Guo, J.H., Russell, R.J., Oakeshott, J.G., Pandey, G., 2011. Degradation of dichloroaniline isomers by a newly isolated strain, *Bacillus megaterium* IMT21. Microbiology 157, 721–726.
Yuan, Y., Zhang, P., Schaffer, A., Schmidt, B., 2017. 3,4-Dichloroaniline revisited: a study on the fate of the priority pollutant in a sediment-water system derived from a rice-growing region in Italy. Sci. Total Environ. 574, 1012–1020.
Zhao, M., Yang, X., Li, Y., Zhang, X., Chang, W., 2001. Spectrophotometric determination of aniline in polluted surface water and wastewater - improved reagents in test kit format. Int. J. Environ. Anal. Chem. 81, 323–330.
Zhu, B., Liu, T., Hu, X., Wang, G., 2013. Developmental toxicity of 3,4-dichloroaniline on rare minnow (*Gobiocypris rarus*) embryos and larvae. Chemosphere 90, 1132–1139.

UNCORRECTED PROOF