보건학 박사학위논문

Effects of aniline and several aniline derivatives on sex hormone regulation and reproduction of adult zebrafish

Aniline 과 유도체가 zebrafish 성체의 성 호르몬 조절 및 생식에 미치는 영향

2019 년 8 월

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Effects of aniline and several aniline derivatives on sex hormone regulation and reproduction of adult zebrafish

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이 논문을 보건학박사 학위논문으로 제출함

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Abstract

Effects of aniline and several aniline derivatives on sex hormone regulation and reproduction of adult zebrafish

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Aniline (ANL) and several aniline derivatives, e.g., 3,4-dichloroaniline (3,4-DCA), 1-naphthylamine (1-NPA), 4,4'-methylenedianiline (4,4'-MDA), and 4- chloroaniline (4-CA) have been widely used in the production of herbicides, pharmaceuticals, cosmetic, dyes, rubber, and adhesives products. These chemicals can easily be released into the environment through industrial and municipal discharges or as degradation byproducts of those materials. Because of the wide range of use and possible exposure to human and environment, these compounds are considered priority pollutants in environmental risk assessment. There are reports on the environmental and human exposure of anilines. Experimental studies and epidemiological observations suggest possible adverse effects of anilines on endocrine and reproduction endpoints. ANL is rapidly metabolized to paracetamol and exhibits similar fetal anti-androgenic effects in male mice. It was also

reported to disrupt testosterone level in the H295R cell line. 3,4-DCA was found to disrupt sex hormone level in tilapia fish in a previous study. Regarding the reproduction, a decrease of the fecundity of fish exposed to ANL and 4-CA was also reported. However, only ANL in cell line and mice and 3,4-DCA in fish were studied to understand endocrine disruption. Moreover, these studies were mostly descriptive and did not focus on the mode of action and effect on the reproduction relating to sex hormone regulation system. So, knowledge of the sex hormone disruption potentials and toxicological effects on reproduction are limited. The aim of this study was to investigate sex hormone disruption potentials of aniline and several aniline derivatives with the effects on the regulatory pathway and effect on the reproduction of zebrafish.

To fulfil the objective of this study we carried out a series of study consisting of two main parts. In the first part (Chapter 2), the objective was to screen the sex hormone disruption effects of aniline and several aniline derivatives using the H295R cell line and zebrafish (*Danio rerio*). Sex hormone levels and steroidogenesis was investigated by using four important steroidogenic genes. In the H295R cell line, 3,4-DCA, 1-NPA and 4,4'-MDA significantly decreased T level with the increase of E2/T ratio. E2 level was not changed after 1-NPA and 3,4-DCA exposure but significantly decreased after 4,4'-MDA exposure. The mRNA expression of *CYP17* was down-regulated and *CYP19A* was up-regulated by both 1-NPA and 3,4-DCA in the cells. All four investigated genes, *StAR*, *CYP17*, *3β-HSD* and *CYP19A* were down-regulated in 4,4'-MDA-exposed cells. In adult male zebrafish upon 14 days exposure to targeted compounds, significant decreases in plasma T and E2 level were observed after 3,4-DCA exposure, but for 1-NPA exposure, the pattern was similar with an inconsistent result in the lower level of exposure concentration. Among the major gonadal

steroidogenic genes, *star* and *cyp17* genes were down-regulated after exposure to 3,4-DCA, while for 1-NPA exposure *3β-hsd* was down-regulated with a significantly decreasing trend of *cyp17* in zebrafish. The steroidogenic genes, i.e., *star*, *cyp17* and *3β-hsd*, are associated with the enzymes which play an important role in the production of T, and *cyp19a* gene in the conversion of T to E2. The transcriptional alteration of these genes may lead to alteration of steroid hormones, and possibly to reproduction changes. Anilines were investigated to understand the effect on sex steroid hormone production and other biological processes, using *in-vitro* and *in-vivo* experiments.

In the second part (Chapter 3), we aimed to understand the effect of 3,4-DCA and 4,4'-MDA on sex hormone regulation through hypothalamus gonadal (HPG) axis with sex hormone and reproduction of adult zebrafish. Both male and female zebrafish were exposed for short term (21 days). A significant decrease of the egg number was observed upon exposure to both the compound. Plasma concentration of both testosterone (T) and 17β-estradiol (E2) level was significantly decreased. In 3,4-DCA, E2/T ratio was decreased in both sex fish whereas E2/T ratio was increased in male and decreased in the female fish upon exposure to 4,4'-MDA. The sex hormone changes was supported by the regulatory changes of the genes along the HPG axis in both sex fish. For example, down-regulation of *star* and *cyp19a* genes in the gonad and upregulation of hypothalamic genes such as *gnrh2* or *gnrh3* genes transcription were observed. Moreover, down-regulation of *ptgs2* gene was detected, suggesting disruption of oocyte maturation and ovulation by the exposure. Our observations indicate that 3,4-DCA and 4,4'-MDA may influence steroid hormone production by altering the major regulatory genes in the HPG axis and eventually influence the reproduction of zebrafish. Consequences of longer-term exposure to this aniline warrant further investigation.

Based on the results of the series of experiments of our study, we can demonstrate that, some of the aniline derivatives e.g., 3,4-DCA, 1-NPA, 4,4-MDA are potential for alteration of sex hormone levels and disrupt the steroidogenesis and as a consequence of effect on the sex hormone balance, 3,4-DCA and 4,4'-MDA have influence on reproduction of zebrafish. 1-NPA was not investigated for the effect on HPG axis and reproduction. It should be done in future for understanding the mode of action and reproductive effect by 1-NPA. Moreover, the effect on longer-term exposure to these anilines warrants further investigation. The findings of this study will be useful for the assessment of the ecological risk of these compounds. It can also keep a great contribution to manage and regulate the safety of anilines in the environment.

Keywords: aniline, endocrine, disruption, sex hormone, dichloroaniline, methylene dianiline, naphthylamine, testosterone, estradiol, H295R cell, zebrafish

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Dedication

I am dedicating this thesis to my beloved wife Farhana Akter Lucky and my lovely children Sameen Sadman Bhuiyan and Shayaan Sabahat Bhuiyan whom has given amplest support to complete my PhD.

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Abbreviation

11-KT: 11 ketotestosterone

1-NPA: 1-naphthylamine

3,4-DCA: 3,4-dichoroaniline

4,4'-MDA: 4,4'-methylenedianiline

4-CA: 4-chloroaniline

ANL: Aniline

AR: Androgen receptor

C*t*: Threshold cycle

CYP11A: Cytochrome P450 side-chain cleavage

CYP17: Cytochrome P450 17

CYP19A: Cytochrome P450 19A

CYP19B: Cytochrome P450 19B

DMSO: Dimethyl sulfoxide

E2: 17β-estradiol

ER: Estrogen receptor

FSH: Follicle stimulating hormone

FSHR: Follicle stimulating hormone receptor

FSHβ: Follicle stimulating hormone β

GnRH: Gonadotropin-releasing hormone

GnRHR: Gonadotropin-releasing hormone receptor

HMGR: Hydroxymethylglutaryl CoA reductase

HSD: Hydroxysteroid dehydrogenase

LH: luteinizing hormone

LHβ: Luteinizing hormone β

StAR: Steroidogenic acute regulatory protein

T: Testosterone

Chapter 1 Introduction

1.1 Properties, usage and production of anilines

Aniline is the parent molecule of the aromatic amines family. It is one of the hundred most important building blocks of many chemicals (Sihtmae et al., 2010). Aniline (ANL) and its derivatives, e.g., 3,4-dichloroaniline (3,4-DCA), 1-naphthylamine (1-NPA), 4,4'-methylenedianiline (4,4'-MDA), and 4- chloroaniline (4-CA) have been widely used in the production of pesticides, pharmaceuticals, cosmetics, dyes, rubber, and adhesives products (Lewis et al., 2013; Mattarozzi et al., 2013; Saleh et al., 2016; Wang et al., 2016). The properties and uses of these chemicals are listed in Table 1.1 and 1.2, respectively.

These five anilines have many common uses and are high volume production chemical in many countries (EU report, 2006; Sihtmäe et al., 2010). In 2016, worldwide aniline production was surpassed by 5.6 million tons (Wang et al., 2016). Other anilines have production or use above 1 million to 10 million pounds in the USA (Di Girolamo et al., 2009; USEPA, 2009). In Korea, all four anilines except 4-CA are high production volume chemicals (NIER, 2018). The production amounts of these chemicals publicly available are listed in Table 1.3.

Table 1.1. Properties of aniline and aniline derivatives.

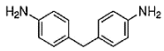
Name	**Abbreviation**	**CAS No**	**Structure**	**Molar mass (g/mole)**	**LogKow**
Aniline	ANL	62-53-3		93.13	0.9
3,4- dichloroaniline	3,4-DCA	95-76-1		162.013	2.69
4,4'-methylene dianiline	4,4'-MDA	101-77-9		198.269	1.59
4-chloroaniline	4-CA	106-47-8		127.57	1.83
1-Naphthylamine	1-NPA	134-32-7		143.19	2.25

Table 1.2. Usage of aniline and its derivatives.

Name	Use	Reference
ANL	Intermediate for the dye, agricultural herbicides or fungicides, polymer, rubber and pharmaceuticals	Wang et al.,2016
3,4-DCA	Intermediate for production of diuron, propanil, and linuron which are used in agriculture and antifouling paint (Diuron), azo dyes, paints, cosmetics.	Saleh et al., 2016; Yuan et al., 2017
4,4'-MDA	The primary raw material for industrial polyurethane flexible and rigid foams, epoxy resins, elastomers, adhesives, binders, coating and paints	Robert et al., 2007; O'Neil, 2006; Lewis, 2007
4-CA	The precursor of antimicrobial and bacteriocide chlorhexidine, pyraclostrobin, anilofos, monolinuron and chlorphthalim; intermediate for urea herbicides and insecticides (e.g., monuron, diflubenzuron), azo dyes, pigments, pharmaceuticals and cosmetics	Lewis, 2001; USEPA, 2005
1-NPA	The precursor of azo dyes (hair dyes), herbicides, and a rubber antioxidant	Mattarozzi et al., 2013; Mar et al., 2006

Table 1.3. Production or consumption of anilines.

Name	Production or amount of use	Reference
ANL	5.6 million tons worldwide in 2016	Wang et al.,2016
	1 billion pounds each year in the USA	Di Girolamo et al., 2009
	190,078 tons produced in Korea in 2007	Ministry of Environment in Korea, 2007
3,4-DCA	13,500-15,500 tonnes/annum in EU in 1996-98	EU report, 2006
	High production volume in Korea (> 1000 tons)	NIER, Korea
4,4'-MDA	>1 million - 10 million in USA in 2002	USEPA, 2009
	212,037 tons produced in Korea in 2007	Ministry of Environment in Korea, 2007
	110,000 tons per year	EC, 2001
	10000-100000 tons per year in EU	ECHA, 2016
4-CA	3000-3300 tons global in 1988; 800-1300 tons India	WHO 2003
	7 metric tons in Germany at 1987	Könnecker et al., 2003
	1000-10000 tons per year in EU	ECHA, 2016
1-NPA	280 million lbs in the USA	Babcock Jr et al., 1993
	High production volume in Korea (> 1000 tons)	NIER, Korea
	100-1000 tons per year in EU	ECHA, 2016

1.2 Environmental occurrence of anilines

These compounds can easily be released into the environment in many diverse ways from the chemical and other industries through effluent or as a breakdown product of those materials such as ANL, 4-CA and 1-NPA from the drug and pesticides, 3,4-DCA from the pesticides (Diuron, linuron and propanil) (Marques et al., 2015; Salvestrini et al., 2002), 4,4'-MDA from the rubber products or polyurethane (Campanella et al., 2015). Because of the wide range of use and possible exposure to human and environment, these compounds are considered priority pollutants in environmental risk assessment (Horie et al., 2017). ANL and 4-CA are classified as hazardous substances in Annex I of Directive 67/548/EEC by the European Commission (Sihtmäe et al., 2010). Some anilines are listed in Table 1.4 as possible metabolites of many pesticides or herbicides.

Table 1.4. Some herbicides and their possible metabolites.

Herbicides	**Possible metabolites**
Chlorobromurin, carboxin, carbetamid, desmediphan, dichlofluanid, fenuron, fenfuram, pencycuron, propham, propachlor, siduron	Aniline
Urox, buturon, monulinuron, diflubenzuron, dimilin, monuron,	4-chloroaniline
Diuron, propanil, linuron, benzoylprop-ethyl, neburon,	3,4-dichloroaniline

Source: Weiss and Angerer, 2002.

Environmental exposure data of these chemicals are limited. However, river water, sediments of many places are found contaminated with a considerable concentration level of these chemicals (Saleh et al., 2016). Agricultural uses of pesticides and wastewater from the industries could be the main source of the contamination (Babcock

Jr et al., 1993). As a building block of many dyes and personal care products, these are also detected in the dyes like hair colour, henna, tattoo and cosmetics (Akyuz and Ata, 2008). Pharmaceuticals can also be a source of anilines in the environment (Kawakami et al., 2010). 1-NPA and 4,4'-MDA can be released from the rubber products under a certain temperature (Campanella et al., 2015). Residual isocyanates remaining in polyurethane adhesives react with the moisture and derived to a primary amine. Furthermore, side reactions during the reaction between isocyanates and polyols can build weak links such as allophanates, biurets and dimers that can dissociate to free isocyanates at temperatures as low as 100 ◦C (Pezo et al., 2008). Thus, the resulting packaging materials built from these plastic multilayers release aromatic amines e.g., 4,4'-MDA which can migrate to the food in contact with them (Pezo et al., 2008). A list of the detection of anilines in the environment and various products are given in Table 1.5. Palmiotto et al. (2001) detected aniline in the indoor air of homes at levels ranging from 5ng/m^3 to about 33ng/m^3, with higher values being observed in the homes of smokers. Aniline was also detected in the air of several public building and in outdoor air, highlighting the ubiquitous presence of aniline in the environment (Palmiotto et al., 2001).

Table 5. Detection of anilines in the environment and various compartments.

Name	Matrix	Sampling time	Detection range	Reference
ANI	Lake water, Bhopal, India		4.46 ± 0.053 nM/L	Shelke et al., 2005
	Water (Buffalo river in the USA)		5.0 mg/L	Bhunia et al., 2003
	Water (Harrach river, Algeria,100 m from medeterian sea)		23.631 µg/mL	Boulahlib et al., 2016
	Water (River, Turkey)		up to 91.03 ng/L	Akyuz and Ata et al., 2006
	Sediment (River, Turkey)		up to 211.36 ng/L	Akyuz and Ata et al., 2006
	Drinking water (Spain)	2010-2011	0.85-25 ng/L	Jurado-Sanchez et al., 2012
	Qinghe river, China		180 µg/L	Zhao et al., 2001
	Water (Rhine river, Netherlands)	2007-2008	0.059-0.075 µg/L	Penders et al., 2012
	Soil (bank of Buffalo river in the USA)		5 ppm	Nelson and Hites, 1980
	Hair dyes and Henna		0.47-8470 µg/g	Akyuz and Ata, 2008
	Dyed hair		9.40-648 µg/g	Akyuz and Ata, 2008
	Dark shades of hair colour		maximum 9681 µg/g	Akyuz and Ata, 2008
	Polyamide cooking utensils (Norway)		82±6 mg/kg	Brede and Skjevrak, 2004
	Spoon		0.33± 1.33 µg/g	Rubio et al., 2014
	Kitchen utensils (84 samples from Ireland)	2009	34.62-100531.98 µg/kg	McCall et al., 2011
3,4-DCA	Water (ports and marinas of Bushehr, Iran)	October 2013	maximum of 390 ng/L	Saleh et al., 2016
	Seawater samples (major harbours of Korea)		13-230 ng/L (Diuron)	Kim et al., 2014
	Seawater (three major bays of Korea)		41-416 ng/L (Diuron)	Kim et al., 2014
	The open water of Jinhae Bay, Korea		39–1360 ng/L (Diuron)	Kim et al., 2014
	Sugarcane soil sample		3.98-4.15 mg/Kg	Felicio et al., 2016

(Continued)

Name	Matrix	Sampling time	Detection range	Reference
3,4-DCA	Wastewater (effluent) (Urban area of Spain)		up to 366 mg/L	Köck-Schulmeyer et al., 2013
	Water (Shoreham Harbour and Brighton Marina, UK, 2004)		Diuron: up to 366 ng/L	Gatidou et al., 2007
	Water and sediment (California marinas USA, CA, 2008)	Summer, 2008	Diuron: <2–68 ng/L	Sapozhnikova et al., 2013
	Water (Japan 2003)		Diuron: 13–350 ng/L	Harino et al., 2005
	River water (Llobregat River basin of Spain)	2010-2011	Diuron: up to 159 ng/L	Masiá et al., 2015
	Water (Crouch Estuary, Essex, Sutton Harbour in the UK)	1998	Diuron: up to 6740 ng/L	Thomas et al.,2001
	Water (Sope Creek and Chattahoochee River, Georgia, USA)	2011-2012	1.8-68.2 ng/L	Hladik and Calhoun., 2012
	Drinking water (Spain)	2010-2011	2.6-180 ng/L	Jurado-Sanchez et al., 2012
4,4'-MDA	Aqueous food simulants		6.8 -184.6 µg/L	Aznar et al., 2009
	Food simulant		0.66-0.75 µg/L	Rubio et al., 2014
	Spoon		0.70-7.42 µg/L	Rubio et al., 2014
	Kitchen utensils (Ireland)	2009	34.62-100531.98 µg/kg	McCall et al., 2011
4-CA	Surface water (Rhein river in Germany)	1990	0.1-1µg/L	Könnecker et al., 2003
	Hair dyes and Henna		0.04-8720 ug/g	Akyuz and Ata, 2008
	Water (River, Turkey)		up to 2.74 ng/L	Akyuz and Ata et al., 2006
	Sediment (River, Turkey)		up to 1.19 ng/L	Akyuz and Ata et al., 2006
	Drinking water (Spain)	2010-2011	5.2-58 ng/L	Jurado-Sanchez et al., 2012
	Lake water, Bhopal, India		2.27 ± 0.01 nM/L	Shelke et al., 2005
	Textile and germants item		0.01 - 100.09 ppm	Kawakami et al., 2010

(Continued)

Name	Matrix	Sampling time	Detection range	Reference
1-NPA	Water (River, Turkey)		upto66.12 ng/L	Akyuz and Ata et al., 2006
	Sediment (River, Turkey)		up to 186.45 ng/L	Akyuz and Ata et al., 2006
	Sediment (Buffalo river in the USA)		7-80 ppb	Nelson and Hites, 1980
	Hair dyes and Henna		0.02-5421 µg/g	Akyuz and Ata, 2008
	Dyed hair		0.04-18.14 µg/g	Akyuz and Ata, 2008

1.3 Human exposure

There are several bio-monitoring studies on anilines (appendix A) where the urinary concentration was detected up to 384 µg/L. Over 90% of the aniline detection frequency was observed in the urine samples collected from 1004 individuals from the general German population. The main routes of aniline exposure in the general population have been postulated to be pesticide residues, pharmaceuticals, colourant used in food, cosmetics, and textiles, and cigarette smoke (German Federal Ministry of Environment 2011). It is believed that aniline and its derivatives are omnipresent in the Western world (Holm et al., 2016). Aniline can be detected as acetaminophen in the human urine because ~78% of the aniline is converted to acetaminophen within 4 hours (Modick et al., 2014). Some other indirect bio-monitoring studies based on the acetaminophen detection in the urine without known aniline exposure or paracetamol intake evident a vast human exposure of aniline (Appendix B).

1.4 Toxicity of anilines

Experimental studies and epidemiological observations suggest possible adverse effects of anilines on endocrine and reproduction endpoints. Aniline was rapidly metabolized to paracetamol in the liver and exhibited fetal anti-androgenic effects in male mice and showed reduced (anogenital distance) AGD (Holm et al., 2015). The testosterone level was increased when exposed to human adrenal carcinoma cell line indicate a sex hormone-disrupting chemical. The same effect was evident in the experimental animal while aniline was exposed to rat (Holm et al., 2015). Other than reproductive toxicity,

aniline shows hepatotoxicity, methemoglobinemia and oxidative stress as common toxicity of amines.

3,4-DCA showed developmental toxicity with a dose-dependent reduced survival, body length and increased malformation after 72 h exposure to embryos and larvae of rare minnow (*Gobiocypris rarus*). All reproductive parameters were strongly affected Daphnia at above a concentration of 9 µg/L of 3,4-DCA (Trubetskova and Lampert, 2002). Sex hormone disruption like estrogenic activity in female and anti-androgenic effect in male Nile Tilapia was also observed (Pereira et al., 2015; 2016).

Reproductive and developmental toxicity were observed while exposed to 4-CA and 1-NPA to adult zebrafish and larvae. The survival and delayed hatching were also observed in 1-NPA exposure. 4,4'-MDA was mostly studied for the hepatotoxicity, genotoxicity and carcinogenesis. Ecotoxicological studies on aquatic organisms are listed in Table 1.6. Some of the aquatic organisms were found sensitive to several aniline derivatives for their survival, growth and reproduction. According to the LOEC (lowest observable effect concentration), the environmentally detected concentration of 3,4-DCA could be dangerous for *Daphnia magna*, *Poecilia reticulate, Danio rerio* and *Oncorhynchus mykiss* (Barata and Baird 2000; Girling et al., 2000; Schäfers and Nagel, 1991). Other toxicological studies on the cell line, mice, rat and fish are listed in Table 1.7.

There is no guideline for the environmental limit of exposure except a guideline for the production of plastics materials and articles in the USA and European Union. USA has banned the aromatic based PU adhesives for the production of multilayer food packages and European legislation has made a limit for the detection of aniline in the food and

food simulant which is 0.01 mg total primary amine as aniline per kg substance (USFDA, 2000; European Commission, 2011).

Table 1.6. Toxicity of anilines on aquatic organisms.

Chemical	Species		End point	Parameter	Exposure time	Results	Reference
ANL	Crustacean	*Daphnia magna*	Immobilization	EC50	48 h	0.13±0.04 mg/L	Sihtmae et al., 2010
		Daphnia magna	Immobilization	EC50	48 h	0.68 mg/L	Abe et al., 2001
		Daphnia magna	Reproduction	EC50	21 days	0.04 mg/L	Gersich and Milazzo, 1988
	Vertebrate	*Oreochromis mossambicus*	Adult mortality	LC50	96 h	69.4 mg/L	Bhunia et al., 2003
		Danio rerio	Mortality	EC20	5 days	18.25 mg/L	Horie et al., 2016
		Brachydanio rerio	Hatching and growth	NOEC	5 days	1.8 mg/L	Van Leeuwen et al., 1990
		Pimephales promelas	Growth	NOEC	7 days	15.7 mg/l	Marchini et_al., 1992
3,4 - DCA	Crustacean	*Daphnia magna*	Immobilization	EC50	3 days	0.0146 mg/L	Barata and Baird 2000
	Vertebrate	*Oncorhynchus mykiss*	Growth	LC50	18 days	0.012 mg/L	Girling et al., 2000
		Poecilia reticulata	Growth	LOEC	42 days	0.002 mg/L	Schäfers and Nagel, 1991
		Danio rerio	Growth	LOEC	42 days	0.02 mg/L	Schäfers and Nagel, 1991
4 - CA	Crustacean	*Daphnia magna*	Immobilization	EC50	48 h	0.31 mg/L	Könnecker et al., 2003
		Daphnia magna	Reproduction	NOEC	21 days	0.01 mg/L	Könnecker et al., 2003
	Vertebrate	*Oryzias Latipes*	Larval growth	MATC	28 days	<2.25 mg/L	Holcombe et al., 1995
		Brachydanio rerio	Mortality	NOEC	3 weeks	1.8 mg/L	Könnecker et al., 2003
1 - NPA	Vertebrate	*Oryzias Latipes*	Mortality	EC50	48 h	7 mg/L	Tonogai et al., 1982
		Oryzias Latipes	Mortality	EC50	24 h	15 mg/L	Tonogai et al., 1982
		Danio rerio	Mortality	LC50	8 days	0.4 mg/L	Horie et al., 2016
4,4' - MDA	Vertebrate	*Danio rerio*	Mortality	LC50	96 hours	65 mg/L	EC, 2001

LC: Lethal concentration; EC: Effective concentration; MATC: maximum acceptable toxicant concentration;
NOEC: No observable effect concentration; LOEC: Lowest observable effect concentration.

Table 1.7. Toxicological studies on anilines.

Chemical name	Studies	Species	Description	End point	Observed result
ANL	Holm et al.,2015	Mice	50, 100 or 150 mg/kg/day paracetamol or equivalent aniline gestation to delivery	Reproductive toxicity (Sex hormone and AGD)	Testosterone level was increased by aniline and decreased by paracetamol and both had an effect on reduction of AGD in male rats after 10 weeks exposure
	Bhunia et al., 2003	Tilapia fish	Aniline, 0.02 - 6.94 mg/L, chronic test for 90 days	Growth and reproductive toxicity	Fecundity, GSI and growth was decreased at 0.05 mg/L
	Chan et al., 2015	*Drosophila melanogaster*	24 hours of exposure	Oxidative stress	Delta genes (Gst D2, Gst D5 and Gst D6) were found to show a peak of up-regulated at 6–8 h of exposure
	Fan et al., 2011	Rat	0.5 mmol/kg/day aniline exposure for 30 days	Nitrosative stress in the spleen, spleen toxicity	Significantly increased iNOS mRNA and protein expression in the spleen; increased reactive nitrogen species formation
	Wang et la., 2015	Rat	0.5 mmol/kg/day aniline exposure for 30 days	Spleen toxicity	Significant increases in the expression of cyclins, CDK1 and aberrant regulation of miRNAs lead to a tumorigenic response in spleen

Continued

Chemical name	Studies	Species	Description	End point	Observed result
	Albert et al., 2013	NCI-H295R adrenocortical human cells	48 hours, 0.0001 M Acetaminophen	Endocrine disruption in adult testis	Anti-androgenic and anti-prostaglandin effects
	Wang et al., 2016	Primary cultured hepatocytes	Exposed to aniline 1.25 to 10.0 g/mL for 24 h	Hepatotoxicity and oxidative stress	ROS, MDA increased; GSH, CAT, SOD decrease; cell viability reduced, apoptotic death induced
	Kristensen et al., 2012	Ex vivo fetal rat testes	Paracetamol at 0.1 µm to 100 µm	Anti-androgenic effect	Testosterone production was inhibited
	Holm et al.,2015	H295R cell line	Aniline for 48 hours	Steroid hormone disruption	Progesterone and testosterone increased, E2 no change
	Holm et al.,2015	H295R cell line	Paracetamol for 48 hours	Steroid hormone disruption	Progesterone and testosterone reduced, E2 increased
3,4-DCA and Diuron	Pereira et al., 2015	Male Nile tilapia	21 days of exposure	Anti-androgenic activities	Reduced T and 11-KT and Gonado somatic index
	Zhu et al., 2013	Rare Minnow	72 hours of exposure	Developmental toxicity	dose-dependent reduced survival, body length and increased malformation
	Scheil et al., 2009	Zebrafish (*Danio rerio*)	11 days subchronic exposure	ELS toxicity with molecular stress response	Elicited deformations at >0.25 mg/l, locomotor activity and mortality were impaired at C0.5 mg/l, Hsp70 level induced
	Li et al., 2003	Crucian carp(*Carassius auratus*)	0.2, 0.4, and 0.8 mg/L for 15 days	Oxidative stress	Produced oxidative stress ; SOD, MDA increased; GSH decreased

(Continued)

Chemical name	Studies	Species	Description	End point	Observed result
	Pereira et al., 2016	Female Nile Tilapia	21-day exposure	Estrogenic activity	Increased E2, gonadosomatic indices and vitellogenic oocytes
	Behrens et al., 2016	*Oyster (Crassostrea gigas)*		Embryotoxicity	No embryotoxicity observed up to 5 µg/L
	Behrens et al., 2016	*Oyster (Crassostrea gigas)*		Genotoxicity	Damages detected from the concentration of 0.05 µg/L and slightly genotoxic
	Carbajal-Hernández et al., 2017	Viviparous fish (*Chapalichthys pardalis*)	21 days subchronic biotoxicity test	Maternal-embryonic metabolic and antioxidant response	Alterations in the antioxidant activity and oxidative damage, embryos and the maternal liver is mostly affected
	Cardone et al., 2008	Lizard (*Podarcis sicula*)	Exposed to Diuron, the parent compound of 3,4-DCA, 3 weeks	Male reproductive toxicity with sex hormone	GSI and plasma testosterone and E2 level was decreased
	Ensenbach and Nagel, 1995	Zebra fish (*Brachydanio Rerio*)	(binary exposure with Lindane)	Early life stage growth (survival, length and weight)	2 µg/L of 3,4-DCA and 40 µg/L of Lindane effect the growth of zebrafish at an early life stage
	Felício et al., 2016	Juvenile male tilapia (*Oreochromis mossambica*)	7 days exposure with 40 ng/L and 200 ng/L of diuron	Steroidogenesis and vitellogenin	Induced vitellogenin by 3,4-DCA and diuron; Alkylphenol and Diuron mixture induced testosterone biosynthesis

(Continued)

Chemical name	Studies	Species	Description	End point	Observed result
	Fernandes et al., 2007	Male Rats	0, 125 or 250 mg/kg fo diuron per dayfor30days	Reproductive effects	No effect in any parameter except the number of fetuses in the litters were reduced
	Guilhermino et al., 1998	Male wester rats	Intra-peritoneal exposure of 3,4-DCA at 0, 81, 162, 324, 486, and 568 mg/Kg of body weight	Biochemical cellular blood parameter	Methemoglobin formation, ED 50 was observed at 224 to 837 mg DCA/Kg
	Zhang and Lin, 2010	Rat	39, 81, 170, and 357 mg/kg of 3,4-DCA exposed for 4 weeks	Effect on Testicle Enzymes	Activities of ALP, ACP, and SDH were increased; LDH, LDH-X, and G6PDH were inhibited; the weight of testis, liver increased
	Ito et al., 2010	Zebrafish adult and embryo	3 months old zebrafish and embryo of 24 hpf exposed for 24 h at 4 μM 3,4-DCA	Ahr/Cyp1a1 pathway responses	*CYP1a1* expression was markedly activated then Ahr in the gill, intestinal epithelia, skin epidermis, and liver parenchyma cell
	Newman et al., 2001	Golden medaka (*Oryzias latipes*) larvae	15 days exposed to Diuron	Developmental toxicity	Delayed time to hatch
	Nebeker et al., 1998	Fathead minnow embryo larvae	7days exposure to Diuron	Growth	Decreased total body length

(Continued)

Chemical name	Studies	Species	Description	End point	Observed result
	Holcombe et al., 1995	Japanese medaka (*Oryzias latipes*)	28 days larval exposure	Survival and growth	Survival and growth was reduced significantly
	Bauchinger and Schmid, 1989	Human lymphocytes	54 or 72 hours of exposure	Genotoxic effect (chromosome aberration assay)	Positive sister-chromatid exchange response in the presence of a metabolic activation system
	Bauchinger and Schmid, 1989	V79 Chinese hamster cells	3 hours of exposure	Genotoxic effect (spindle disturbance)	Concentration dependent spindle disturbance with aneuploidy by interaction with the mitotic apparatus
4,4'-MDA	Bailie et al., 1993	Rat	24 hours; several doses of 25-225 mg/kg were orally administered	Hepatotoxicity	Serum alanine amino transferase, glutamyl transferase, bilirubin increased; Necrosis and portal oedema found in the histogram
	Dugas et al., 2004	Male and Female Rat	25 mg for 17-22 weeks	Hepatotoxicity	Concentric fibrosis around bile ducts of the liver was noted, vascular medial hyperplasia was also prominent
	Kwon et al., 2008	Mouse	10-100 mg/kg	Hepatotoxicity and oxidative stress	Increased liver-toxicity-related enzymes in blood and induced bile-duct cell injury, followed by regeneration

(Continued)

Chemical name	Studies	Species	Description	End point	Observed result
	Parodi et al., 1983	Male Swiss mice	9 or 18 mg/kg body weight	Genotoxicity	Dose-dependent sister chromatid exchange was observed
	Lamb et al., 1986	Rat	103 weeks; 150-300 mg/kg dose	Carcinogenesis	Lower body weight, low water intake and nonneoplastic effects observed in the thyroid
	Martelli et al., 2002	Rat and human hepatocyte	10 to 180 µM; 4-20 hours exposure	Genotoxicity	Dose-dependent DNA lesion
4-CA	Bresch et al., 1990	Zebrafish (*Brachydanio rerio*)	1, 0.2, and 0.04 mg/L	Reproductive and developmental toxicity	Reduced egg production, fertilization and growth
	Okazaki et al., 2003	Rat	(15 days exposure with 300 mg/Kg wt)	Neurotoxicity	Reduced response of hind limb extensor thrust, gait abnormality in the open field, grip strength and rearing episodes
	Holcombe et al., 1995	Japanese medaka (*Oryzias latipes*)	28 days larval exposure	Survival and growth	Survival and growth was reduced significantly at 8.29 mg/L
	Horie et al., 2016	Zebrafish (Danio rerio) embryo larvae	8 days post fertilization exposure	Survival and growth	ZF embryo larval reduced survival and hatching delay at 10.5 mg/L
1-NPA	Horie et al., 2016	Zebrafish (*Danio rerio*) embryo larvae	8 days post fertilization exposure	Survival and growth	ZF embryo larval reduced survival and hatching delay at 0.3 mg/L

Regarding the endocrine disruption by aniline and aniline derivatives, there is a knowledge gap on the sex hormone disruption and related mechanism of aniline and its derivatives both in *in-vitro* and *in-vivo*. Firstly, a limited number of anilines have been investigated like *in-vitro* hormone disruption only reported for aniline, and fish hormone disruption is only reported for 3,4- DCA. Secondly, limited understanding of the mode of action on the endocrine system because the previous studies were mostly descriptive and none of the studies investigated the effect on the regulatory pathway of sex steroid production. Finally, the consequences of the effect on sex hormone level are not well known. Moreover, it is totally unknown whether they have effects on the endocrine system of zebrafish which is a well-accepted model organism for testing the effect of endocrine disrupting chemicals.

Therefore, this study was set to fill the gap of knowledge of the effect of aniline and its derivatives on sex hormone with the effect on the regulatory pathway of steroidogenesis and consequently on reproduction.

1.5 Study design and objectives

The aim of the study was to investigate sex hormone disruption potentials of aniline and aniline derivatives and associated mechanisms in order to provide information on the toxicological effects of anilines on the endocrine system and consequences of zebrafish. The obtained information from this study will be useful in ecological risk assessment of this group of compounds. It will also be useful to manage and regulate anilines safety in the environment.

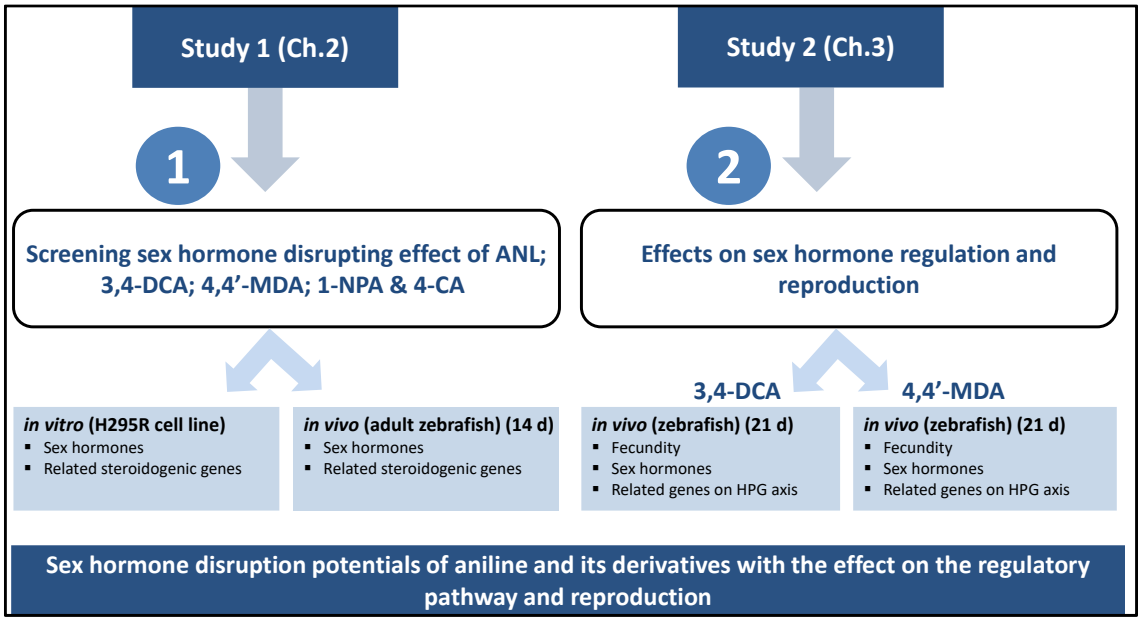


Figure. 1.1. Study design to investigate sex hormone disruption potentials of aniline and aniline derivatives, effect on regulatory pathway, and reproduction.

This study consisted of two main parts (Figure 1.4). The first part (Chapter 2) contained two separate experiments. In one experiment, we screened the sex hormone disrupting potentials of ANL, 3,4-DCA, 4,4'-MDA, 1-NPA, and 4-CA using *in vitro* cell line (H295R cell line). In the second experiment, the selected anilines in H295R cell i.e, 3,4-DCA, 4,4'-MDA, and 1-NPA were then further screened to investigate the effect in *in vivo* using zebrafish (*Danio rerio*) model. In both cell line and organism level, the effect on sex hormone alteration and major steroidogenic genes were analyzed to understand the mechanism of action. In the second part of this study (Chapter 3) was also consisted of two separate experiments where the aim was to understand the effect of 3,4-DCA and 4,4'-MDA on sex hormone regulation through hypothalamus gonadal (HPG) axis and reproduction of adult zebrafish. Both male and female zebrafish were exposed for short

term (21 days). Combining all the experiments, our results demonstrate that the investigated aniline derivatives have sex hormone disruption potentials. The findings of the present study can contribute to the toxicological information of targeted aniline derivatives with the mechanism of action and consequences.

Chapter 2 Endocrine disruption by several anilines and related mechanisms in a human adrenal H295R cell line and adult male zebrafish

2.1 Introduction

Aniline is a parent compound of the aromatic amines family. It is one of the important building blocks of numerous chemicals. There are several hundreds of aniline derivatives. 3,4-dichloroaniline (3,4-DCA), 1-naphthylamine (1-NPA), 4,4'-methylenedianiline (4,4'-MDA) and 4-chloroaniline (4-CA) are some of the top used aniline derivatives. Aniline and these derivatives have been widely used as precursors or intermediates for the production of many herbicides, dyes, pharmaceuticals, cosmetics, rubber, and adhesives products (Lewis, 2007; Mattarozzi et al., 2013; Saleh et al., 2016; Wang et al., 2016). Several anilines are identified as high volume production chemicals in several countries including USA and EU member states (Di Girolamo et al., 2009; European Commission, 2006, 2012; Sihtmäe et al., 2010; USEPA, 2009). Only aniline production in 2016 was more than 5.6 million tons (Wang et al., 2016).

As precursors or intermediates of the production of many dyes, adhesives and personal care products, aniline and its derivatives can be released from various products. For example, 1-NPA can be released from henna, tattoo, hair colour, and cosmetics (Akyuz and Ata, 2008). Rubber products can release 1-NPA and 4,4'-MDA under a certain temperature and 4,4'-MDA can be formed from residual isocyanates remaining in polyurethane adhesive (Pezo et al., 2012). Pharmaceuticals can be another source of the

anilines in the environment as aniline is used for the preparation of antipyretics, analgesics, anti-allergics, and vitamins (Kawakami et al., 2010). Moreover, these compounds can be released into the environment as breakdown products, and these examples are 4-CA and 1-NPA from the dyes, herbicides, and pesticides (Babcock Jr et al., 1993; Guzman Mar et al., 2006), 3,4-DCA from the herbicides e.g., diuron, linuron, and propanil (Marlatt and Martyniuk, 2017; Saleh et al., 2016), and 4,4'-MDA from polyurethane and rubber products (Campanella et al., 2015; Rubio et al., 2014).

While the information on the environmental levels of many anilines are relatively limited, a few anilines have often detected in river water, and sediments around the world (Boulahlib et al., 2016; Jurado-Sanchez et al., 2012; Saleh et al., 2016; Zhao et al., 2001). In Sope Creek and Chattahoochee River water in the USA, 3,4-DCA was detected up to 68.2 ng/L (US Geological Survey, 2012). In the sediment of a river in Zonguldak industrial area in Turkey, 1-NPA was detected up to a seasonal mean concentration of 186.45 ng/kg (Akyuz and Ata, 2006). The parent compound of 3,4-DCA, e.g., diuron, was detected up to 230 ng/L in the major harbours of Korea and up to 1360 ng/L in a fishing port of Jinhae Bay of Korea (Kim et al., 2014). Because of the wide range of source and detection in the environment, the compounds are considered for toxicological investigation.

Several experimental and epidemiological studies suggest that aniline may cause possible adverse effects on the endocrine system and reproduction. Following exposure to aniline, fetal male rats showed reduced anogenital distance suggesting anti-androgenic effects (Holm et al., 2015). In the human adrenal carcinoma cell line (H295R), exposure to aniline lead to increased testosterone levels (Holm et al., 2015). Similar toxicities have been also reported for a few other organisms. In *Daphnia*, aniline

influenced reproductive parameters following 21 days of exposure (Trubetskova and Lampert, 2002). Similar toxic effects have been reported for other anilines. In Nile tilapia (*Oreochromis niloticus*), exposure to diuron metabolite i.e., 3,4-DCA led to increasing 17β-estradiol in female and decreasing testosterone levels in male fish (Pereira et al., 2015; 2016). Following exposure to 1-NPA in embryo larvae of zebrafish, toxic effects on survival and development were observed (Horie et al., 2017). In a rare minnow (*Gobiocypris rarus*) larvae, a 72 h exposure to 3,4-DCA exposure caused a dose-dependent decrease of growth and increase of malformation. While evidence indicating endocrine disruption potentials of aniline and its derivatives are accumulating, knowledge on underlying mechanisms is still limited. Moreover, only very few aniline derivatives have been studied, and therefore there is a significant information gap on the toxicity of other commonly used and detected aniline derivatives.

The objective of this study was to evaluate endocrine disrupting potentials of major aniline derivatives and to identify possible underlying mechanisms, using both in vitro and in vivo experiments. For this purpose, the human adrenal H295R cell line and adult male zebrafish were employed. H295R cells have been widely used for the screening of the endocrine disruption potentials of chemicals specially focusing on changes in steroidogenesis (Hecker et al., 2011; Hecker and Giesy, 2008; Hilscherova et al., 2004; Jo et al., 2014; Kraugerud et al., 2011; Sanderson et al., 2002; Sanderson, 2006). Zebrafish is chosen for further screening in the organism level. Zebrafish have been widely used to investigate the effects of environmental chemicals on sex hormone disruption and to characterize the underlying mechanisms at an organism level (Ji et al., 2013; Liu et al., 2012; Ma et al., 2012; Segner, 2009; Segner et al., 2003;Sohn et al.,2016; Wang et al., 2015). The results of this study will help identify aniline

derivatives of potential toxicological concern and stimulate further studies on endocrine disruption consequences of aniline derivatives.

2.2 Materials and methods

2.2.1 Chemicals

ANL (CAS No. 62-53-3, purity: ≥99.5%), 3,4-DCA (CAS No. 95-76-1, purity: ≥98%), 1-NPA (CAS No. 134-32-7, purity: ≥99%), 4,4'-MDA (CAS No. 101-77-9, purity: ≥97%) and 4-CA (CAS No. 95-76-1, purity: ≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). As a solvent, Dimethyl sulfoxide (DMSO) was used for both *in vitro* and *in vivo* studies. The final concentration of the solvent (is used for dissolving the compounds as a carrier) in the exposure media (water) was 0.005% (v/v) for zebrafish exposure and 0.1% (v/v) in the cell media for H295R cell assays.

2.2.2 H295R cell culture and exposure

Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12) (Sigma D-2906; Sigma-Aldrich) were used for the H295R cell line. The cells were cultured at 37 ◦C in a 5% CO2 atmosphere (Hilscherova et al., 2004), and the media were renewed every other day. The exposure concentration range was determined at 1.0, 10.0, and 100.0 mg/L for all five test chemicals, based on the results of the WST-1 cell proliferation assay (Appendix C; Figure. A1) (Roche Applied Science, Mannheim, Germany). As a positive control, Forskolin was exposed to the cells along with the tested chemicals. Each exposure concentration contained three replicates. The 24-well plate was used for exposure. The cells were seeded at a density of 3.0×10^5 cells/mL, and

after 24 h of incubation, the cells were exposed to the chemical for another 48 h. After the exposure, the culture medium was collected for sex steroid hormone measurement, and the cells were collected for quantification of the gene expression. The H295R cell line assay was repeated for three times independently and the results were combined to conduct statistical analysis.

2.2.3 Zebrafish culture and exposure

About six months old adult male zebrafish (*Danio rerio*, wild type) were obtained from a commercial vendor (Green Fish, Seoul, Korea). According to the H295R cell results and previous experimental reports on male fetal mice (Holm et al., 2015), male sex hormone seemed to be most affected. Therefore, male zebrafish were chosen for the fish screening test. The fish were acclimated in the laboratory in-house culture environment at least for seven days before the experiment. Three test chemicals, i.e., 3,4-DCA, 1-NPA, and 4,4'-MDA were exposed to the fish at 6 concentration levels including water and solvent controls. For each concentration and control, four replicates with 4 male fish each in a 2 L beaker were used. The exposure concentrations for each chemical were determined at sub-lethal levels, i.e., 0.024, 0.12, 0.6, and 3.0 mg/L for 3,4-DCA; 0.04, 0.2, 1.0, and 5.0 mg/L for 1-NPA; and 0.2, 1.0, 5.0, and 25 mg/L for 4,4'-MDA, based on preliminary study. The study was carried out for 14 days according to the OECD test guideline No. 204 for fish prolonged toxicity test: 14-day study (OECD, 1984). The fish were maintained at 26 ± 1˚C under 14:10 h light: dark photoperiod, and fed with freshly hatched *Artemia nauplii* twice in a day.

The exposure media (>90%) was replaced daily with newly prepared test media. During renewal, both new and old exposure media were measured for water quality parameters

including temperature, pH, dissolved oxygen, and conductivity every alternative day. After the 14 day exposure, the fish were sacrificed, and blood samples were collected from the caudal vein using a capillary tube. To obtain sufficient volume for hormone measurement, the blood samples from four fishes were pooled, and plasma was separated by centrifugation (8000 rpm for 10 min at 4˚C). The plasma was stored at −80˚C until hormone analysis. For fish, testes were collected from three male zebrafish from each replicate and pooled for measurement of transcriptional changes. For each treatment or control, a total of four replicates were measured and used for statistical analysis. The collected testes samples were stored at −80˚C until analysis by qPCR analysis for major steroidogenic genes.

2.2.4 Chemical analysis in fish exposure water

The concentrations of 1-NPA, 3,4-DCA, and 4,4-MDA in the fish exposure water were measured at the beginning and after 24 h of exposure. Exposure water was collected on three different days from each replicate of each concentration on each day i.e., one sample from each beaker. Briefly, 1 mL of the water sample was filtered with a 0.2 μm syringe filter. After that, the sample was diluted with water to ensure the concentration fell within the calibration curve range. All three chemicals were 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). Chemicals were separated using ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm) column. The injection volume was 5 μL and the flow rate was 0.3 mL/min. Water and Methanol were used as mobile phase with 20:80 ratios in isocratic mode. Total run time was 3 minutes. Analytical conditions using UHPLC-

MS / MS are shown in Table 2.1 and MS / MS parameters are shown in Table 2.2. The limit of detection was 0.13 ng/mL for 1-NPA, 0.22 ng/mL for 3,4-DCA, and 0.15 ng/mL for 4,4-MDA.

Table 2.1. Analytical conditions using UHPLC-MS/MS.

Parameter		**Condition**	
UHPLC	Column	ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	
	Mobile phase	A: Water B: Methanol	
	Isocratic	Time (min) 0 3 B (%) 80 80	
	Flow rate	0.3 mL/min	
	Injection volume	5 μL	
MS/MS	Ionization mode	ESI positive	
	Curtain gas (CUR)	25	
	Collision gas (CAD)	8	
	Ion spray voltage (V)	5500	
	Temperature	550	
	Ion source gas 1	40	
	Ion source gas 2	60	

Table 2.2. Retention time and MRM parameters.

Compound	RT(min)	Parent ion (Q1)	Daughter ion (Q3)	DP(volts)	CE(volts)	CXP(volts)
1-NPA	0.96	144.09	127.00	91	33	4
	0.96	144.09	77.00	91	45	8
3,4-DCA	0.97	162.10	127.00	91	31	10
	0.97	162.10	109.00	81	21	18
4,4-MDA	0.92	199.11	106.20	106	33	10
	0.92	199.11	77.10	106	73	6

*DP: declustering potential (V), CE: collision energy (V), CXP: collision cell exit potential (V)

2.2.5 Measurement of sex hormones and gene expressions

In both H295R cell medium and zebrafish, sex hormones of 17β-estradiol (E2) and testosterone were measured using enzyme-linked immunosorbent assay (ELISA) with commercial kits (Cayman Chemical; 17β-estradiol [Cat No. 582251] and testosterone [Cat No. 582701]). Hormones were extracted from 500 µL of H295R cell medium or 10 µL of fish plasma. The samples were diluted with 400 µL of ultrapure water. The diluted samples were centrifuged at 2000 g for 10 min after adding 2 mL of diethyl ether, and then the upper layer was collected. The extraction by diethyl ether was done twice. The sample was evaporated under nitrogen flow and was reconstituted with 300 µL of EIA buffer for the H295R cell line and 120 µL for the zebrafish for hormone measurement using ELISA kit (Ji et al., 2010). For fish, plasma samples from four male fish in each replicate were pooled and measured for hormones. For each treatment or control, four replicates were measured for the hormones.

Measurement of four major steroidogenic genes was carried out by quantitative real-time PCR (qRT-PCR). For this purpose, either H295R cells or fish organ samples were

homogenized and total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA, USA). The complementary DNA was synthesized for all samples using an iScriptTM cDNA synthesis kit (BioRad, Hercules, CA, USA) from 100 ng/µL of extracted RNA. After the extraction of RNA and synthesis of cDNA, quality and concentration of RNA and cDNA were confirmed by absorbance ratio of 260/280 nm using Epoch (BioTek, Winooski, VT, USA). The qRT-PCR sample was prepared by adding 18 µL of premix and 2 µL of cDNA sample in a 96 well plate. The premix contained 10 µL of Light Cycler DNA Master SYBR Green I mix (Roche Diagnostics Ltd., Lewes, UK), 1.8 µL of forward and reverse primer (10 pmol) and 4.4 µL of nuclease-free water. The qRT-PCR was carried out using Light Cycler 480 (Roche Applied Science, Indianapolis, IN, USA). Thermal cycling was 95◦C for 10 min followed by denaturation at 95◦C for 10 s, annealing at 60◦C for 20 s, extension at 72◦C for 20 s with a total 40 PCR cycles. After the final amplification, melting curve analyses were carried out to identify the desired PCR products from the primer dimers or contaminants. Two pseudo-replicates were analyzed of each true replicates. The qRT-PCR was carried out using Light Cycler 480 (Roche Applied Science, Indianapolis, IN, USA). The relative expression level of mRNA of each targeted gene was normalized with that of the reference gene (*β-actin* in both H295R and zebrafish) and calculated by threshold cycle (C*t*) number using ΔΔCt method (Livak and Schmittgen, 2001). The primer sequences of targeted genes in H295R and zebrafish in this study were used according to the previous study by Ji et al. (2013). The primer sequences of targeted genes in H295R and zebrafish are listed in Table 2.3.

Table 2.3. Primer sequences used for the quantitative RT-PCR analysis for H295R cell and zebrafish samples.

Assay	Gene name	Accession No.	Description	Sequence (5'-3')
In vitro H295R	*β-actin*	NM_001101	Forward	CAC TCT TCC AGC CTT CCT TCC
			Reverse	AGG TCT TTG CGG ATG TCC AC
	StAR	NM_000349	Forward	GTCCCACCCTGCCTCTGAAG
			Reverse	CATACTCTAAACACGAACCCCACC
	3βHSD	NM_000198	Forward	TGCCAG TCT TCA TCTACACCAG
			Reverse	TTCCAGAGGCTCTTCTTCGTG
	CYP17	NM_000102	Forward	AGCCGCACACCAACTATCAG
			Reverse	TCACCGATGCTGGAGTCAAC
	CYP19A	NM_000103	Forward	AGGTGCTATTGGTCATCTGCTC
			Reverse	TGGTGGAATCGGGTCTTTATGG
In vivo zebrafish	*β-actin*	NM_131031	Forward	TGCTGTTTTCCCCTCCATTG
			Reverse	TCCCATGCCAACCATCACT
	star	NM_131663	Reverse	GGTCTGAGGAAGAATGCAATGAT
			Reverse	CCAGGTCCGGAGAGCTTGT
	3βhsd	AY279108	Forward	AGGCACGCAGGAGCACTACT
			Reverse	CCAATCGTCTTTCAGCTGGTAA
	cyp17	AY281362	Forward	TCTTTGACCCAGGACGCTTT
			Reverse	CCGACGGGCAGCACAA
	cyp19a	AF226620	Forward	GCTGACGGATGCTCAAGGA
			Reverse	CCACGATGCACCGCAGTA

2.2.6 Statistical analysis

The normality of distribution and homogeneity of variances were assessed by Shapiro-Wilk's test and Levene's test, respectively. When the data did not follow a normal distribution, log transformation was performed to make them normally distributed. To compare for differences among the treatments one-way analysis of variance (ANOVA) followed by Dunnett's t-test was performed using SPSS 23.0 for Windows (SPSS Inc., Chicago, IL, USA). All results are presented as mean ± standard deviation (SD), and $P<0.05$ was considered statistically significant.

2.3 Results

2.3.1. Concentrations of chemicals in fish exposure media

Measured concentrations of 3,4-DCA, 1-NPA, or 4,4'- MDA for nominal experimental concentrations are shown in Table 2.4. The average of the measured concentrations for the fresh (0 h) and old (24 h) media of the highest experimental concentrations of 3,4-DCA, 1-NPA, or 4,4'-MDA were about 60%, 50%, or 80% of the nominal concentrations, respectively (Table 2.4). In the lower experimental concentrations, measured concentrations for 1-NPA and 4,4'-MDA were less than 50% of the nominal concentrations, while those for 3,4-DCA were greater than 100% of the nominal concentration. Because of relatively huge differences between nominal and measured concentrations, the measured concentrations, i.e., averages of the measured concentrations for the fresh and the 24 h old media were used for presentation and interpretation of the results.

Table 2.4. Measured concentrations (mg/L) for nominal experimental concentrations of 3,4-DCA, 1-NPA, or 4,4'-MDA in the exposure media at 0 and 24 h of exposure.

3,4-DCA				1-NPA				4,4'-MDA			
Nominal	Measured			Nominal	Measured			Nominal	Measured		
	0 h	24 h	Average		0 h	24 h	Average		0 h	24 h	Average
0.00 (C)	0.00	0.00	0.00	0.00 (C)	0.00	0.00	0.00	0.00 (C)	0.00	0.00	0.00
0.00 (SC)	0.00	0.00	0.00	0.00 (SC)	0.00	0.00	0.00	0.00 (SC)	0.00	0.00	0.00
0.024	0.030	0.041	0.035	0.040	0.014	0.015	0.015	0.20	0.091	0.056	0.074
0.12	0.11	0.16	0.13	0.20	0.15	0.13	0.14	1.0	0.67	0.50	0.58
0.60	0.44	0.33	0.38	1.0	1.2	0.50	0.83	5.0	5.4	3.9	4.6
3.0	2.1	1.8	1.9	5.0	5.6	1.6	3.6	25	25	20	22

C: Control; SC: Solvent control. Average: average of the measured concentrations for the fresh (0 h) and the 24 h old media. Each measured concentrations are an average of 12 indepent samples collected throughout the exposure (N=12).

2.3.2. Responses in H295R cells by aniline derivatives

2.3.2.1. Concentration of sex hormones

In the H295R cell line, three aniline derivatives (3,4-DCA, 1-NPA and 4,4'-MDA) among five tested compounds showed a significant dose-dependent decrease of T (Testosterone) concentrations along with the increase of E2/T ratio (Figure 2.1). E2 level was not changed after 3,4-DCA and 1-NPA exposure but significantly decreased after 4,4'-MDA exposure (Figure 2.1). Forskolin, a positive control chemical used in the H295R cell line assay showed a significant increase in both T and E2 concentrations (Figure 2.2). However, ANL and 4-CA did not show any changes in the hormone level (Appendix D). Not any significant difference in hormone concentrations were observed between the control and solvent control (Data not shown).

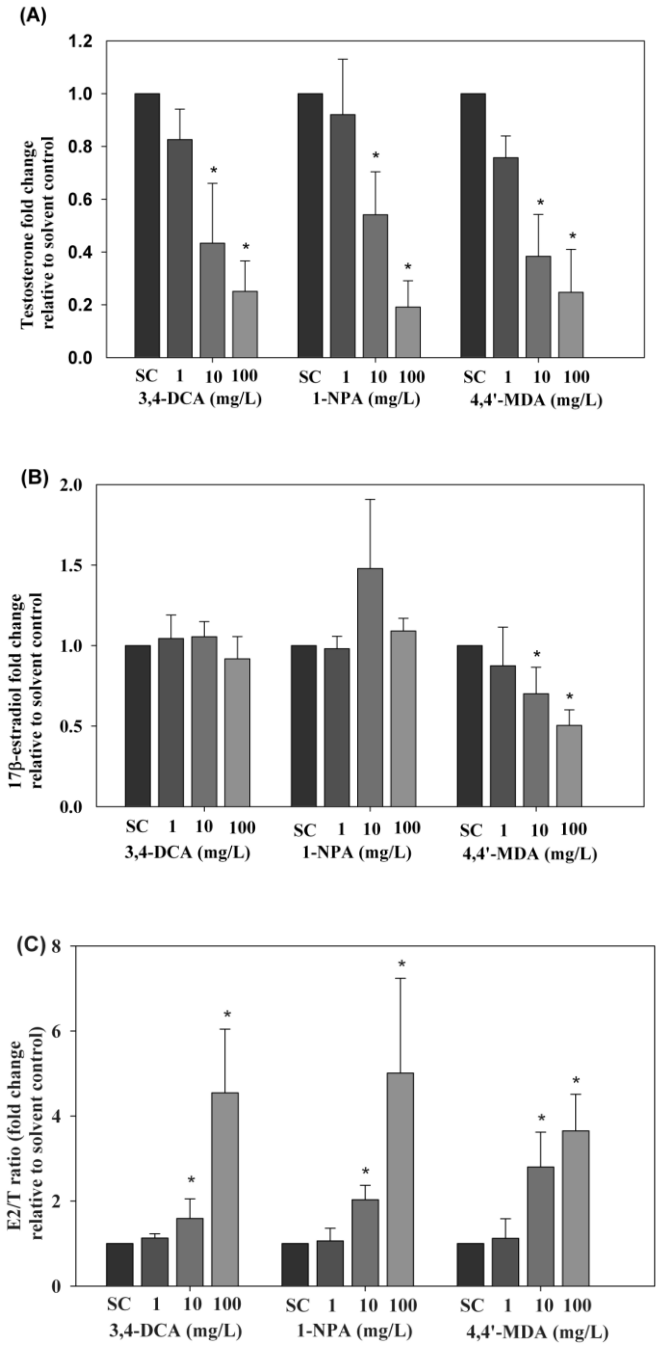


Figure 2.1. (A) Testosterone (T), (B) 17β-estradiol (E2), and (C) E2/T ratio measured in the cultured medium of H295R cells after 48 h exposure to 3,4-DCA, 1-NPA and 4,4'-MDA. Results are shown as mean ± SD of three independent experiments. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.1% DMSO).

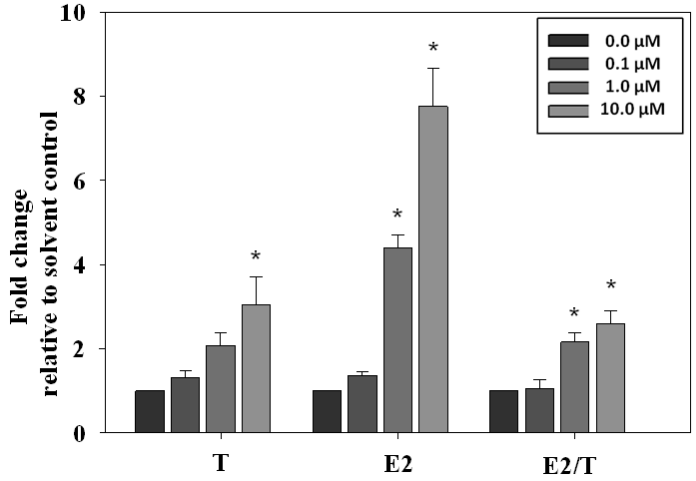


Figure 2.2. Testosterone (T), 17β-estradiol (E2), and E2/T ratio measured in the cultured medium of H295R cells after 48 h exposure to forskolin (0.1-10 µM). Results are shown as mean ± SD of three independent experiments. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.1% DMSO).

2.3.2.2. mRNA expression level of steroidogenic genes

The *CYP17* gene was down-regulated and *CYP19A* gene was up-regulated by both 1-NPA and 3,4-DCA in the H295R cells (Figure. 2.3). After exposure to 4,4'-MDA, *StAR*, *CYP17* and *CYP19A* were down-regulated (Figure 2.3). However, the *3βHSD* gene was not altered by any test chemicals. All four targeted genes showed significant up-regulation by the positive control chemical, forskolin (Figure 2.4). However, ANL and 4-CA did not show any changes in the targeted steroidogenic gene expression (Appendix E).

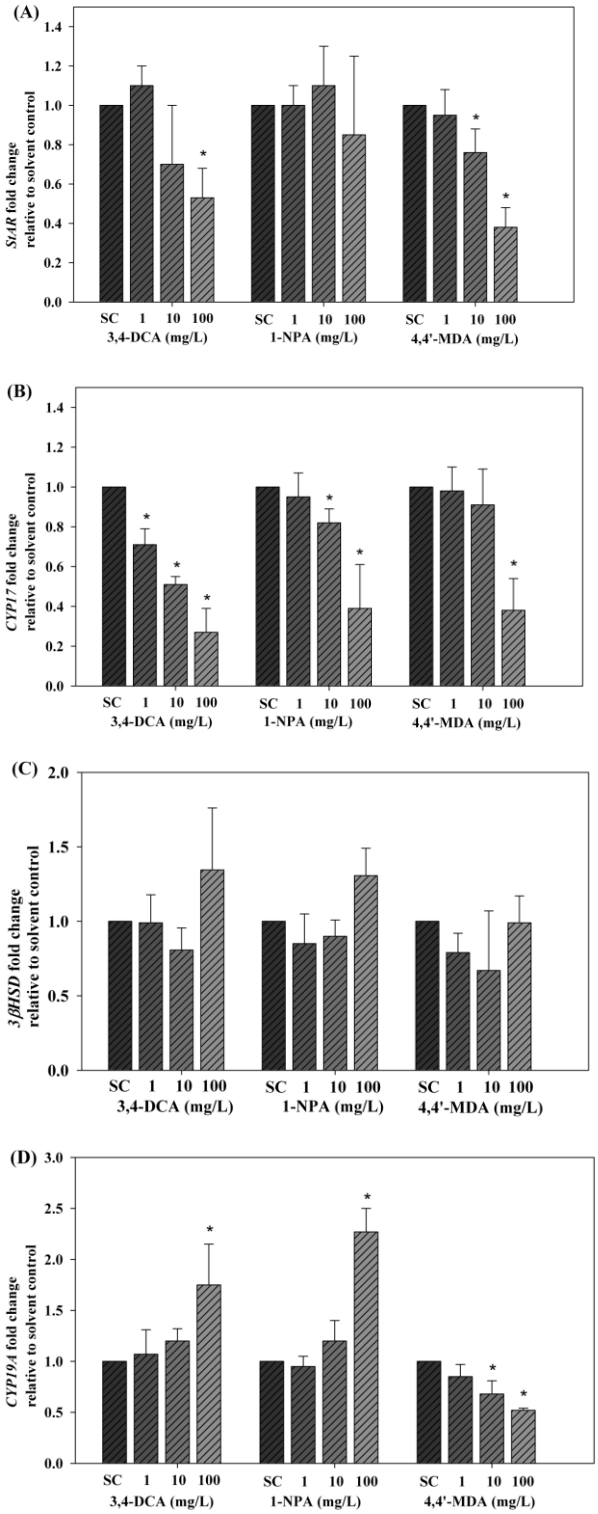


Figure 2.3. mRNA expression level of (A) *StAR*, (B) *CYP17*, (C) *3βHSD* and (D) *CYP19A* gene in H295R cell after 48 h exposure to 3,4-DCA, 1-NPA and 4,4'-MDA.

Results are shown as mean ± SD of three independent experiments. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.1% DMSO).

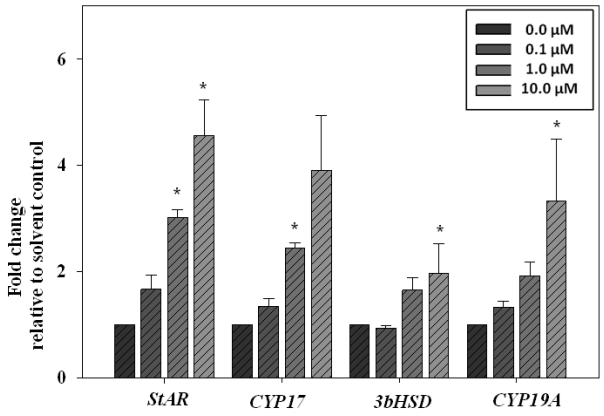


Figure 2.4. mRNA expression level of *StAR*, *CYP17*, *3βHSD,* and *CYP19A* gene in H295R cell after 48 h exposure to forskolin (0.1-10 μM). Results are shown as mean ± SD of three independent experiments. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.1% DMSO).

2.3.3. Responses in adult male zebrafish by aniline derivatives

2.3.3.1. Plasma sex hormones

Only three (3,4-DCA, 1-NPA and 4,4'-MDA) among the five targeted chemicals which showed a significant effect in the H295R cell line were exposed to zebrafish. All three exposed chemicals influenced the sex hormone balances of the adult male zebrafish leading to higher relative estrogenicity, i.e., higher E2/T ratio. Significant decreases of both T and E2 were observed by 3,4-DCA and 4,4'-MDA, and the extent of decrease was much greater for T (Figure 2.5). While the hormonal changes by 1-NPA were not

consistent, and even showed a seemingly increasing pattern of both sex hormones at lower levels of exposure, E2/T ratio showed a similar pattern of increase.

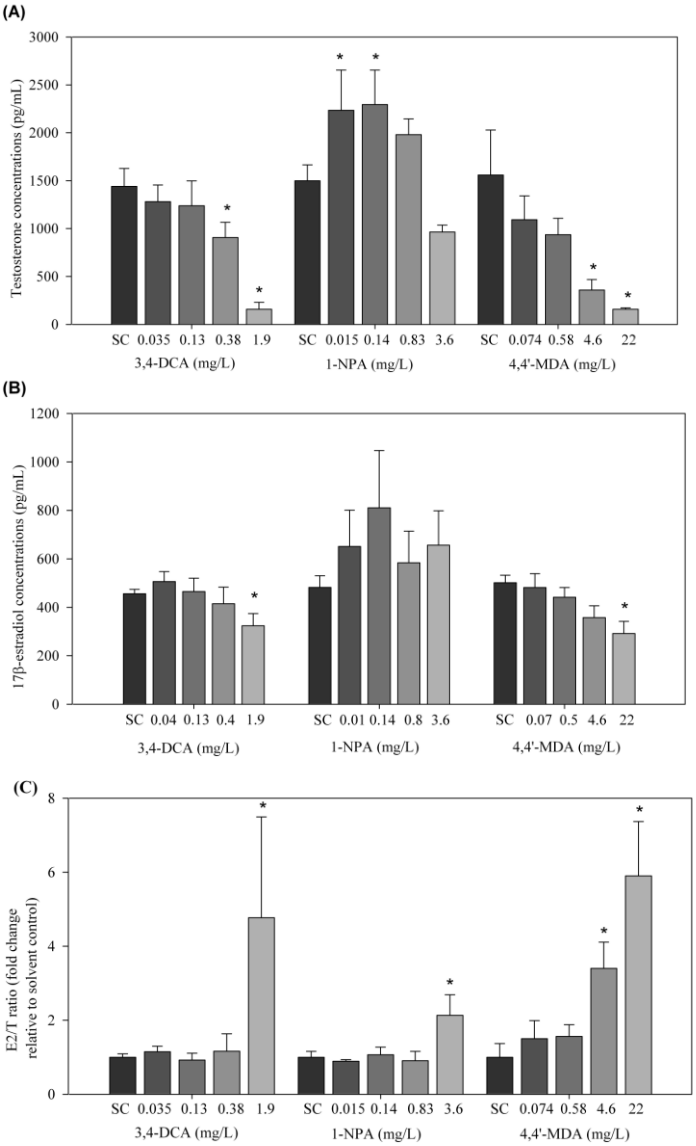


Figure 2.5. Plasma concentration changes of (A) Testosterone (T), (B) 17β-estradiol (E2), and (C) E2/T ratio measured in male zebrafish after 14 d of exposure to 3,4-DCA, 1-NPA and 4,4'-MDA. Results are shown as mean ± SD of four replicates for each concentration. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.1% DMSO).

2.3.3.2. Gonadal steroidogenic genes

2.3.3.2. Gonadal steroidogenic genes

Among the studied gonadal steroidogenic genes, both *star* and *cyp17* genes were down-regulated after exposure to 3,4-DCA or 4,4'-MDA. For 1-NPA, *3β-hsd* and *cyp17* genes were down-regulated with a significant up-regulation of *star* gene at the lowest concentration level (0.015 mg/L) (Figure 2.6 A). The *cyp19A* gene was not influenced by the exposure to the tested anilines but showed a significant up-regulation in the 4,4'-MDA exposure group at 4.6 mg/L level (Figure 2.6).

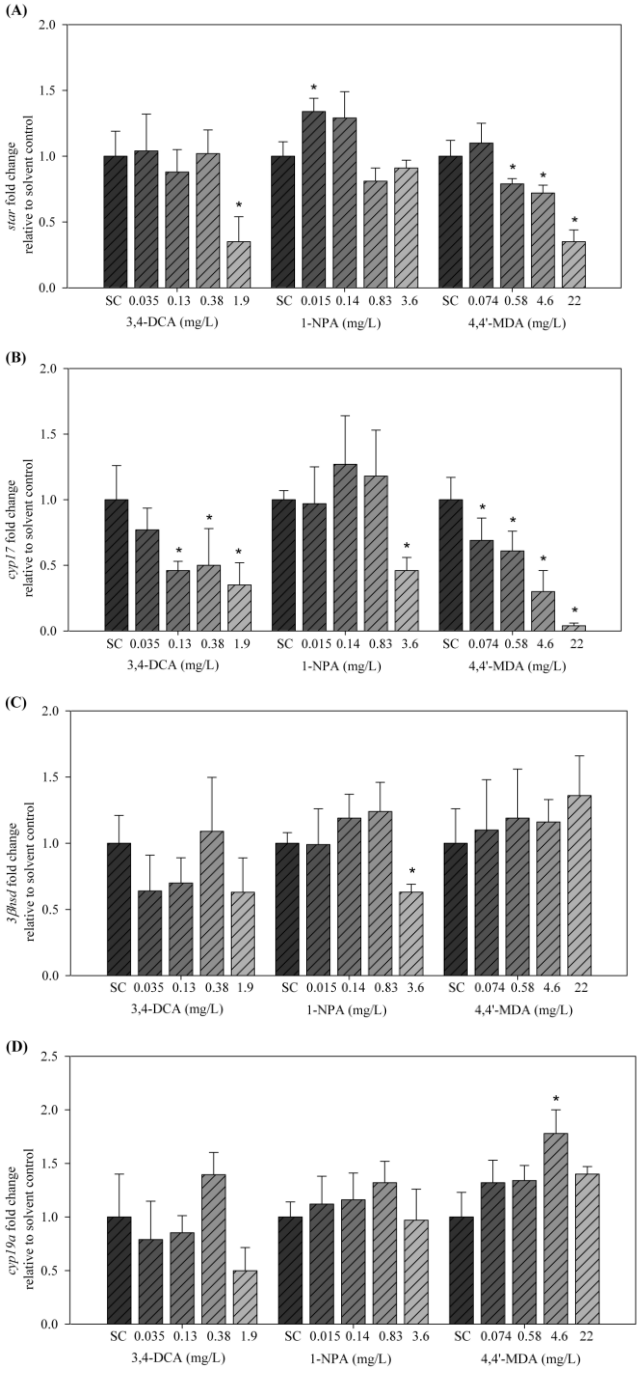


Figure 2.6. mRNA expression level of (A) *star*, (B) *cyp17*, (C) *3βhsd* and (D) *cyp19A* gene in male zebrafish gonad after 14 d exposure to 3,4-DCA, 1-NPA and 4,4'-MDA. Results are shown as mean ± SD of four replicates for each concentration. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.1% DMSO).

2.4 Discussion

A significant dose-dependent decrease of T concentrations along with the increase of E2/T ratio by three aniline derivatives (3,4-DCA, 1-NPA and 4,4'-MDA) among five tested anilines were observed in the H295R cells (Figure 2.1) indicates endocrine disruption potential of these three anilines toward greater E2 to T ratio. ANL and 4-CA did not show any effect on the hormonal changes and steroidogenic targeted gene expression. The increased relative estrogenicity was mostly due to a significant reduction of T levels by exposure to the tested anilines (Figure 2.1.A). Significant down-regulation of major steroidogenic genes such as *StAR* and *CYP17* shows that the decreased T levels may be due to reduced steroidogenesis. Down-regulation of *StAR* and *CYP17* genes (Figure 2.3) may decrease cholesterol uptake and androstenedione synthesis, respectively which lead to reduced production of steroid hormones. The *StAR* gene plays an important role in which protein is responsible for the transport of cholesterol at the starting of the steroidogenic pathway into the mitochondrial membrane (Clewell et al., 2010). The enzyme encoded by the *CYP17* gene is responsible for the conversion of progesterone to androstenedione.

Up-regulation of *CYP19A* gene in the H295R cells observed following exposure to 3,4-DCA and 1-NPA could be interpreted as increased conversion of T to E2 (Figure. 2.1D). Aromatase, the product of the transcription of the *CYP19A* gene, catalyzes the conversion of T to E2 (Hilscherova et al., 2004; Trant et al., 2001). Therefore, the up-regulation of *CYP19A* may lead to enhancement of the conversion of T to E2, which in turn can decrease T levels. Significant up-regulation of the *CYP19A* gene may explain the E2 levels which were not affected, even though several genes in the up-stream of the

steroidogenic pathaway were down-regulated. Decreased E2 levels following exposure to 4,4'-MDA (Figure. 2.1B) can be also explained, in this context, by down-regulation of *CYP19A* gene (Figure. 2.1D). The observations in H295R cells clearly show that the three aniline derivatives could alter the hormonal balances toward decreasing T levels, through modulating the steroidogenic pathway.

To date, endocrine disruption potential of aniline derivatives has seldom been reported, except for aniline. Holm et al. (2015) reported that aniline could increase T synthesis without E2 level change in H295R cells. In the same study, authors also showed that paracetamol, one of the major metabolites of aniline, could decrease T levels and increase E2 levels in the cells through alteration of the steroidogenic pathway, e.g, down-regulation of *CYP17* gene and up-regulation of *CYP19A* gene. For other anilines, however, sex hormone disruption potentials and steroidogenic alteration have never been investigated in the cells. Our observations in the H295R cell study indicate that several major aniline derivatives could influence sex hormone balances towards decreased T levels, through altering the steroidogenic pathway.

The observations in H295R cells were supported by the observations in the zebrafish, which has been widely used to investigate the effects of environmental chemicals on sex hormone disruption and to understand the mechanisms (Ji et al., 2013; Liu et al., 2012; Ma et al., 2012; Sohn et al., 2016; Wang et al., 2015) (Figure 2.5 and 2.6). Employing adult male zebrafish, we found the consistent direction of sex hormone disruption, i.e., increased E2/T ratio (Figure 2.5.C). Among three tested anilines in zebrafish, 3,4-DCA and 4,4'-MDA caused a significant decrease of T levels (Figure 2.5.A). Even though E2 levels were also decreased (Figure. 2.5B), E2/T ratio increased significantly, because the extent of T decrease was much greater than that of E2 decrease. Decreases of both sex

hormones in the adult male fish, similar to the case of H295R cells, could be explained by significant down-regulation of key steroidogenic genes of *star* and *cyp17* following exposure to 3,4-DCA and 4,4'-MDA (Figure 2.6.A, B). Unlike H295R cells, adult male zebrafish exposure did not cause notable up-regulation of *cyp19a* gene (Figure 2.6.D), and therefore E2 levels decreased slightly (Figure 2.5.B). To date, only 3,4-DCA has been reported for endocrine disruption in fish. Following exposure to 3,4-DCA, T was decreased in male tilapia and E2 was increased in female tilapia fish (Pereira et al., 2015; 2016). The observations in the male tilapia fish are the same as those observed in the present study in the male zebrafish. However, in the tilapia fish study, regulatory changes of the related steroidogenic genes were not evaluated.

The hormonal imbalance observed in the adult male zebrafish was in line with the observations from the H295R cell, except for an inconsistent T and E2 level in the lower level exposure to 1-NPA. Lower experimental concentrations of 1-NPA, i.e., 0.015 or 0.14 mg/L, caused up-regulation of *star* gene (Figure 2.6.A), and an increase of T (Figure 2.5.A). However, at the highest concentration, i.e., 3.6 mg/L, those of *3β-hsd* and *cyp17* were down-regulated. The product of *3β-hsd* is essential for the conversion of 17α-OH-progesterone to 17α-OH-progesterone which in turn convert to androstenedione. Therefore, the down-regulation of *3β-hsd* gene possibly leads to reduced sex hormone production. The up-regulation of *star* gene expression at a low level can explain the induced T level by 1-NPA at the lower level. Even though the extent of change was the least among the three tested aniline derivatives, 1-NPA exposure also led to increased E2/T ratio (Figure 2.5.C). While it is clear that 1-NPA disrupts sex hormone balance in both cell line and the fish, but the mechanisms of action seem to be different between the human adrenal cells and the adult male

zebrafish. The aromatase gene, *CYP19A*, was significantly up-regulated in the H295R cell while no changes were observed in the male zebrafish, upon exposure to 1-NPA. The regulation of steroidogenic enzymes can vary between the cell lines and fish, therefore leading to different responses (Baker, 2001; Villeneuve et al., 2007). In addition, sex related difference between the male fish and the adrenal cell line of a female human, i.e., H295R cell, may also in part explain the observed difference in steroidogenic enzyme regulation.

Our observations showed that several anilines such as 3,4-DCA, 1-NPA, and 4,4'-MDA can disrupt hormone balances in male zebrafish, possibly through alteration of steroidogenic pathway. However, this study has several limitations. First, only T was measured in the fish plasma. While T is an important precursor of active androgen in fish, 11- ketotestosterone (11-KT) is considered as better indicator of male sex hormone in fish (Schulz et al., 2010). Secondly, we tested only male zebrafish in the present study, therefore, potential effects on female fish are remained to be investigated. In addition, we measured only several major genes in the steroidogenic pathway, therefore other modes of action that may lead to sex hormone disruption could not be tested. Finally, the experimental concentrations which are orders of magnitude higher than those detected in the environment should be noted (Akyuz and Ata, 2006; Boulahlib et al., 2016; Jurado-Sanchez et al., 2012; Saleh et al., 2016; Zhao et al., 2001). Therefore, further studies employing environmental relevant concentrations with long-term exposure design are warranted to understand ecological consequences of aniline compounds in the water.

In conclusion, considering the experimental limitations and our observations in both H295R cells and adult male zebrafish clearly demonstrate that the three tested aniline

derivatives have a potential to disrupt sex hormone balance, i.e., decrease of T, possibly through alteration of some steroidogenic genes. The consequences of the endocrine disruption caused by exposure to aniline derivatives, especially for a longer period of time, including adverse reproduction or birth effects, warrant further investigations.

Chapter 3 Effects of 3,4-DCA and 4,4'-MDA on sex hormone regulation and reproduction of adult zebrafish

3.1 Introduction

Aniline is a parent molecule of aromatic amines family, and has 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: 3,4-DCA has been used as precursors or intermediates for many herbicides, dyes, and paints (Mattarozzi et al., 2013; Saleh et al., 2016; Yuan et al., 2017), and 4,4'-MDA for industrial polyurethane, flexible and rigid foams, elastomers, adhesives, binders, coating, and paints (Lewis, 2007; Mattarozzi et al., 2013). Both 3,4-DCA and 4,4'-MDA are classified as high volume production chemicals in many countries including Korea, USA, and EU (Di Girolamo et al., 2009; European Commission, 2006, 2012; Ministry of Environment in Korea, 2007; Sihtmäe et al., 2010; USEPA, 2009). 3,4-DCA has relatively high water solubility about 580 mg/L at 20˚C (IHCP, 2006; Zhu et al., 2013). For 3,4-DCA, while complete bacterial degradation is possible, it occurs very slowly (Yao et al., 2011). In contrast, 4,4'-MDA binds covalently with the organic matters in the soil and sediments, and hence is not readily degradable in the environment (ATSDR, 1998). The half-lives for 3,4-DCA and 4,4'-MDA in the environment are estimated at about 6-18 days and 4-190 days, respectively (IHCP, 2006; USEPA, 2009; Zhu et al., 2013).

3,4-DCA is a common precursor or intermediate or, or at the same time, degradation product of many herbicides, mainly linuron, diuron, and propanil (Crossland, 1990). It can easily be released into the environment during the production and use of herbicides

(EC, 2006), and therefore frequently detected in the environment especially in the aquatic environment (Giacomazzi and Cochet, 2004; Hladik and Calhoun., 2012; IHCP, 2006a, 2006b; Jurado-Sanchez et al., 2012; Saleh et al., 2016; Yao et al., 2011). In the USA, 3,4-DCA was detected in the Sope Creek and Chattahoochee River water at up to 68.2 ng/L (US Geological Survey, 2012). Moreover, diuron, a parent compound of 3,4-DCA, was detected in the harbour 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). Simiarly, 4,4'-MDA can released into the environment through industrial effluents or can be leached out from the consumer materials such as rubber products and adhesives, during 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). However, information on the environmental levels of 4,4'-MDA is scarce than other aniline derivatives. Human biomonitoring for European Union (HBM4EU) has included 4,4'-MDA as a priority group for biomonitoring (HBM4EU, 2017).

3,4-DCA has been reported for higher toxicity than parent herbicides for the aquatic organism and endanger development and propagation of organisms in the aquatic system (Bozena and Danuta, 1998; Palau-Casellas and Hutchinson, 1998; Ramos et al., 2002). In *Daphnia magna*, 3,4-DCA can influence reproduction rate and age at first reproduction at the concentration as low as 9 μg/L (Trubetskova and Lampert, 2002). In freshwater rotifer *Brachionus calycforus*, significant decreases of net reproductive rate, generation time and reproductive value were observed at 2.5 mg/L 3,4-DCA (Ferrando et al., 1993). In a larval rare minnow (*Gobiocypris rarus*), a dose-dependent growth decrease and malformation increase were observed upon exposure to 3,4-DCA. While basic eco-toxicological information on 3,4-DCA is accumulating, relatively little is

known about potential sex hormone disruption or reproduction effects of 3,4-DCA. This compound has been reported for the hormonal disruption in Nile tilapia (*Oreochromis niloticus*), i.e., estrogenic effects in female and anti-androgenic effects in male fish (Pereira et al., 2015; 2016). In contrast, 4,4'-MDA is classified as a possible human carcinogen (Group 2B) by IARC (US DHHS, 1993). In rats and mice, exposure to 4,4'-MDA exposure led to several types of tumours and damages in the liver and thyroid, mineralization in kidneys, and reduced body weight (ATSDR, 1998).

Experimental evidences that suggest endocrine disruption potentials and associated consequences of anilines are available (Holm et al., 2015). In a 14 days toxicity test with adult male zebrafish, significant decreases of T and E2 levels along with down-regulation of some steroidogenic genes were observed following exposure to 3,4-DCA and 4,4'-MDA (Bhuiyan et al., 2019). However, detailed information on the sex hormone disruption of these aniline derivatives, e.g., steroidogenic regulation, is largely unknown. In fish, sex hormone balance and eventually reproduction rely on regulation by hypothalamic-pituitary-gonadal (HPG) axis (Ji et al., 2013; Xu et al., 2017). Therefore, the effects of 3,4-DCA and 4,4'-MDA on the steroidogenesis and reproduction are of interest.

The purpose of this study was to investigate the effects of 3,4-DCA and 4,4'-MDA on sex hormone regulation and reproduction of adult zebrafish (*Danio rerio*). Zebrafish has been widely used to investigate the effects of environmental chemicals on sex hormone disruption and to understand the mechanisms (Ji et al., 2013; Liu et al., 2012; Ma et al., 2012; Segner, 2009; Shon et al., 2016; Wang et al., 2015). The results of this study will help to understand the potential toxicological concern and attract follow-up

investigations on ecological consequences of endocrine disruption and reproduction effect of 3,4-DCA and 4,4'-MDA.

3.2 Material and Methods

3.2.1 Chemicals

3,4-DCA (CAS No. 95-76-1, purity: ≥98%) and 4,4'-MDA (CAS No. 101-77-9, purity: ≥97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was used as a solvent for this study. The final concentration of the solvent in the exposure media was 0.005% (v/v) for zebrafish exposure.

3.2.2 Zebrafish culture and exposure

Both male and female adult zebrafish (*Danio rerio*) were purchased from a commercial vendor (Green Fish, Seoul, Korea). The fish was about six months old. They were acclimated in the laboratory at least for fifteen days of in-house culture environment before the experiment. The exposure concentrations for 3,4-DCA were water control (C), solvent control (SC), 0.024, 0.12, and 0.6 mg/L and for 4,4'-MDA were C, SC, 0.2, 1.0, and 5.0 mg/L based on the preliminary study. The OECD test guideline 229 for fish short term reproduction assay was followed, with minor modifications on the fish sex ratio and water renewal (OECD, 2009). The culture conditions were maintained at 26 ± 1˚C under 14:10 h light: dark photoperiod, and freshly hatched *Artemia nauplii* were fed twice a day. The exposure media were renewed daily with new test solutions, and the water quality indicators including temperature, pH, dissolved oxygen, and conductivity were monitored routinely.

Before the exposure is, the pairs of fish were allowed to mate for 7 days to confirme similar fecundity throughout the mating pairs. For exposure, eight replicates per treatment or control were prepared with two male and two female fish in a replicate (Figure. 3.1). During the mating, eggs were counted every day (n=8 per treatment). At the conclusion of the 21-day exposure, fish were sacrificed, and a blood sample was collected from the caudal vein using the capillary tube. Then, blood of four fishes from two beakers of a given treatment was pooled to make one measurement (n=4 per treatment or control). From pooled blood, plasma was separated by centrifugation (8000 rpm for 10 min at 4˚C), and stored at -80˚C until hormone analysis. Moreover, brain and testis were collected from three fish per treatment, and pooled by sex (n=4 per treatment). These isolated organs were stored at -80˚C until further analysis.

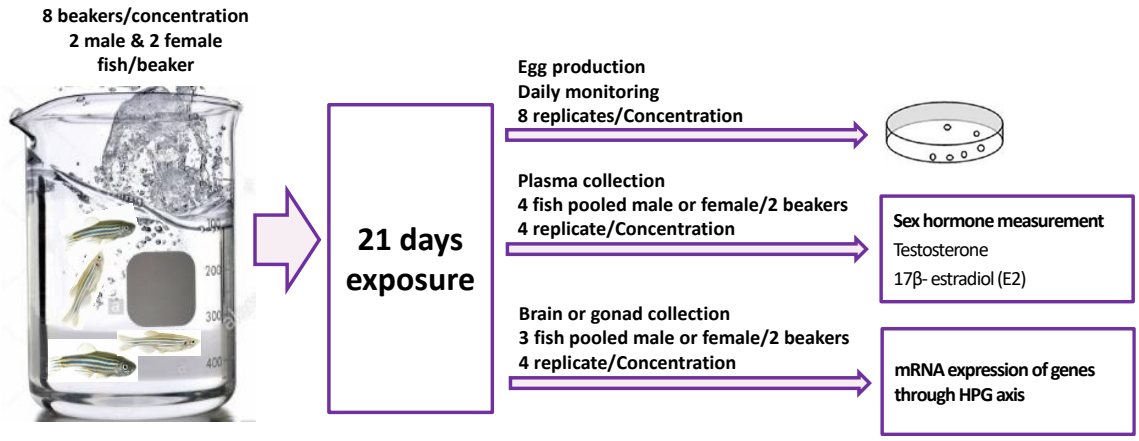


Figure 3.1. Experimental design to investigate the effects of 3,4-DCA and 4,4'-MDA on sex hormone regulation and reproduction of adult zebrafish.

3.2.3 Chemical analysis in fish exposure media

The exposure media in this experiment was renewed daily. The concentrations of 3,4-DCA and 4,4'-MDA in the fish exposure media (water) were measured at the beginning (0 h) and after the 24 h of the exposure (Table 2.4). The limit of detection for 3,4-DCA and 4,4'-MDA were 0.22 ng/mL and 0.15 ng/mL, respectively.

3.2.4 Measurement of sex hormones and gene expressions

Both 17β-estradiol (E2) and testosterone were measured in the plasma of male and female zebrafish. Commercial kits employing enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical; 17β-estradiol [Cat No. 582251] and testosterone [Cat No. 582701]) were used for hormone measurement. For this purpose, first, hormones were extracted from 10 µL of fish plasma: The plasma was diluted to 400 µL with ultrapure water. Briefly, 2 mL of diethyl ether, an extraction solvent, was added to the diluted samples and centrifuged at 2100 g for 10 min. The upper layer of the centrifuged sample

was collected. This extraction procedure was repeated twice with diethyl ether. Then, the extraction solvent of the entire samples was evaporated under nitrogen flow. The dried samples were diluted with 120 µL EIA buffer and used for the hormone measurement (Ji et al., 2010).

The mRNA expression of major genes through hypothalamus pituitary-gonadal (HPG) axis was measured by quantitative real-time PCR (qRT-PCR). For tissue samples including brain and gonad, homogenization was performed before the total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA, USA). The complementary DNA was synthesized from 100 ng/µL of extracted RNA for all samples using an iScriptTM cDNA synthesis kit (BioRad, Hercules, CA, USA). The quality and concentration of extracted RNA and synthesized cDNA were confirmed by Epoch (BioTek, Winooski, VT, USA) at the absorbance ratio of 260/280 nm. Light Cycler 480 (Roche Applied Science, Indianapolis, IN, USA) was employed for qRT-PCR. *β-actin* was used as a reference gene and the relative expression level of mRNA of each target gene was normalized with that of the reference gene. The expression level was calculated by the threshold cycle (C*t*) number using the ΔΔCt method (Livak and Schmittgen, 2001). The primer sequences of targeted genes of zebrafish used in this study were chosen based on our previous study (Ji et al., 2013), and this information is shown in Table 3.1.

Table 3.1. Primer sequences used for the quantitative RT-PCR analysis for zebrafish samples.

Gene name	Accession No.	Description	Sequence (5'-3')
β-actin	NM_131031	Forward	TGCTGTTTTCCCCTCCATTG
		Reverse	TCCCATGCCAACCATCACT
gnrh2	AY657018	Forward	CTGAGACCGCAGGGAAGAAA
		Reverse	TCACGAATGAGGGCATCCA
gnrh3	NM_182887	Forward	TTGCCAGCACTGGTCATACG
		Reverse	TCCATTTCACCAACGCTTCTT
gnrhr1	NM_001144980	Forward	ACCCGAATCCTCGTGGAAA
		Reverse	TCCACCCTTGCCCTTACCA
gnrhr2	NM_001144979	Forward	CAACCTGGCCGTGCTTTACT
		Reverse	GGACGTGGGAGCGTTTTCT
gnrhr4	NM_001098193	Forward	CACCAACAACAAGCGCAAGT
		Reverse	GGCAACGGTGAGGTTCATG
fshβ	NM_205624	Forward	GCTGTCGACTCACCAACATCTC
		Reverse	GTGACGCAGCTCCCACATT
lhβ	NM_205622	Forward	GGCTGCTCAGAGCTTGGTTT
		Reverse	TCCACCGATACCGTCTCATTTA
cyp19b	AF183908	Forward	GTCGTTACTTCCAGCCATTCG
		Reverse	GCAATGTGCTTCCCAACACA
erα	NM_152959	Forward	CAGACTGCGCAAGTGTTATGAAG
		Reverse	CGCCCTCCGCGATCTT
er2β	NM_174862	Forward	TTCACCCCTGACCTCAAGCT
		Reverse	TCCATGATGCCTTCAACACAA
ar	NM_001083123	Forward	TCTGGGTTGGAGGTCCTACAA
		Reverse	GGTCTGGAGCGAAGTACAGCAT
fshr	NM_001001812	Forward	CGTAATCCCGCTTTTGTTCCT
		Reverse	CCATGCGCTTGGCGATA
lhr	AY424302	Forward	GGCCATCGCCGGAAA
		Reverse	GGTTAATTTGCAGCGGCTAGTG
hmgra	BC155135	Forward	GAATCCACGGCCTCTTCGT
		Reverse	GGGTTACGGTAGCCACAATGA
hmgrb	NM_001014292	Forward	TGGCCGGACCGCTTCTA
		Reverse	GTTGTTGCCATAGGAACATGGA
star	NM_131663	Reverse	GGTCTGAGGAAGAATGCAATGAT
		Reverse	CCAGGTCCGGAGAGCTTGT
cyp11a	NM_152953	Forward	GGCAGAGCACCGCAAAA
		Reverse	CCATCGTCCAGGGATCTTATTG
3βhsd	AY279108	Forward	AGGCACGCAGGAGCACTACT
		Reverse	CCAATCGTCTTTCAGCTGGTAA
cyp17	AY281362	Forward	TCTTTGACCCAGGACGCTTT

		Reverse	CCGACGGGCAGCACAA
17βhsd	AY306005	Forward	TGCATCTCGCATCAAATCCA
		Reverse	GTCCAAGTTCCGCATAGTAGCA
cyp19a	AF226620	Forward	GCTGACGGATGCTCAAGGA
		Reverse	CCACGATGCACCGCAGTA
ptgs2	AY028585	Forward	TGGATCTTTCCTGGGTGAAGG
		Reverse	GAAGCTCAGGGGTAGTGCAG

3.2.5 Statistical analysis

Shapiro-Wilk's test and Levene's test were used for assessment of normality of distribution and homogeneity of variances of the data, respectively. Log transformation was performed when the data did not follow a normal distribution. To compare the differences among the treatments including the control, one-way analysis of variance (ANOVA) followed by Dunnett's t-test were performed. All the statistics were carried out using SPSS 23.0 for Windows (SPSS Inc., Chicago, IL, USA). Results are presented as mean ± standard deviation (SD). The $P<0.05$ was considered statistically significant.

3.3 Results

3.3.1. Chemical measurement in exposure media

In the lower experimental concentrations, the measured concentrations were less than 50% of the nominal concentrations for 4,4'-MDA. In contrast, for 3,4-DCA, the measured concentrations were >100% than those of nominal concentrations (Table 3.2). The average measured concentrations of the media at 0 h and 24 h were 63% and 92%, respectively. For simplicity, the averages of initial and 24 h water measurements were used for presenttion and interpretion of the results throughout this chapter.

Table 3.2. Measured concentrations (mg/L) of 3,4-DCA and 4,4’-MDA in the exposure water at 0 h and 24 h of exposure.

3,4-DCA	Nominal		0.00 (C)	0.00 (SC)	0.024	0.12	0.60
	Measured	Initial (0 h)	ND	ND	0.030	0.11	0.44
		Old (24 h)	ND	ND	0.04	0.16	0.33
		Average	0.00	0.00	0.035	0.13	0.38
4,4'-MDA	Nominal		0.00 (C)	0.00 (SC)	0.20	1.0	5.0
	Measured	Initial (0 h)	ND	ND	0.091	0.67	5.4
		Old (24 h)	ND	ND	0.056	0.50	3.9
		Average	0.00	0.00	0.074	0.58	4.6

C: Control; SC: Solvent control. Average: average of the measured concentrations for initial (0 h) and the old (24 h) exposure media (water). Each measured concentrations are an average of 12 indepent samples collected throughout the exposure.

3.3.2. Effects on reproduction

Egg production per spawning events significantly decreased at the highest exposure level of 3,4-DCA and 4,4’-MDA (Figure 3.2A). The cumulative number of eggs as well as spawning events per breeding tank also significantly decreased at the highest exposure level of each chemical (Figure 3.2B, 3.2C). Between the control and solvent control, difference in egg production was insignificant (Data not shown).

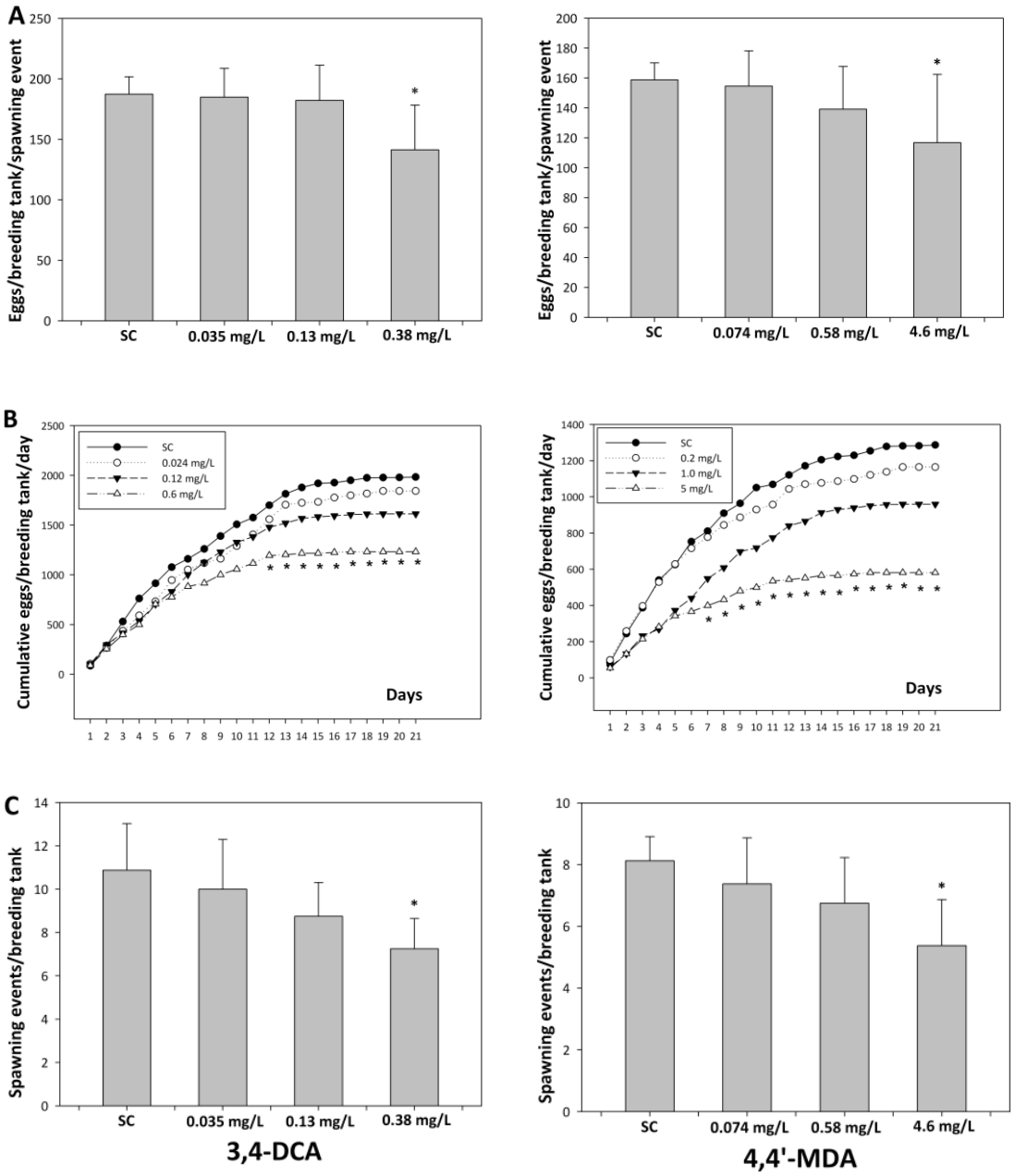


Figure 3.2. Effects of 3,4-DCA and 4,4'-MDA on the reproduction of zebrafish. A: eggs/per breeding tank/spawning; B: Cumulative eggs/breeding tank/day); C: Spawning events/breeding tank. One breading tank represents two pairs of zebrafish and n=8. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.005% DMSO).

3.3.3. Plasma sex hormones

The sex hormone levels of both male and female zebrafish were altered following exposure to 3,4-DCA and 4,4'-MDA. In the male fish, significant decreases of both T and E2 level were observed at 0.38 mg/L 3,4-DCA and 0.58 mg/L and 4.6 mg/L 4,4'-MDA (Figure 3.3). In addition, a decrease of E2 level was observed at 4.6 mg/L 4,4'-MDA. In female fish, decrease of both T and E2 were 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, in the male fish, the extent of T decrease was much greater, and therefore higher E2/T ratio was observed (Figure 3.3).

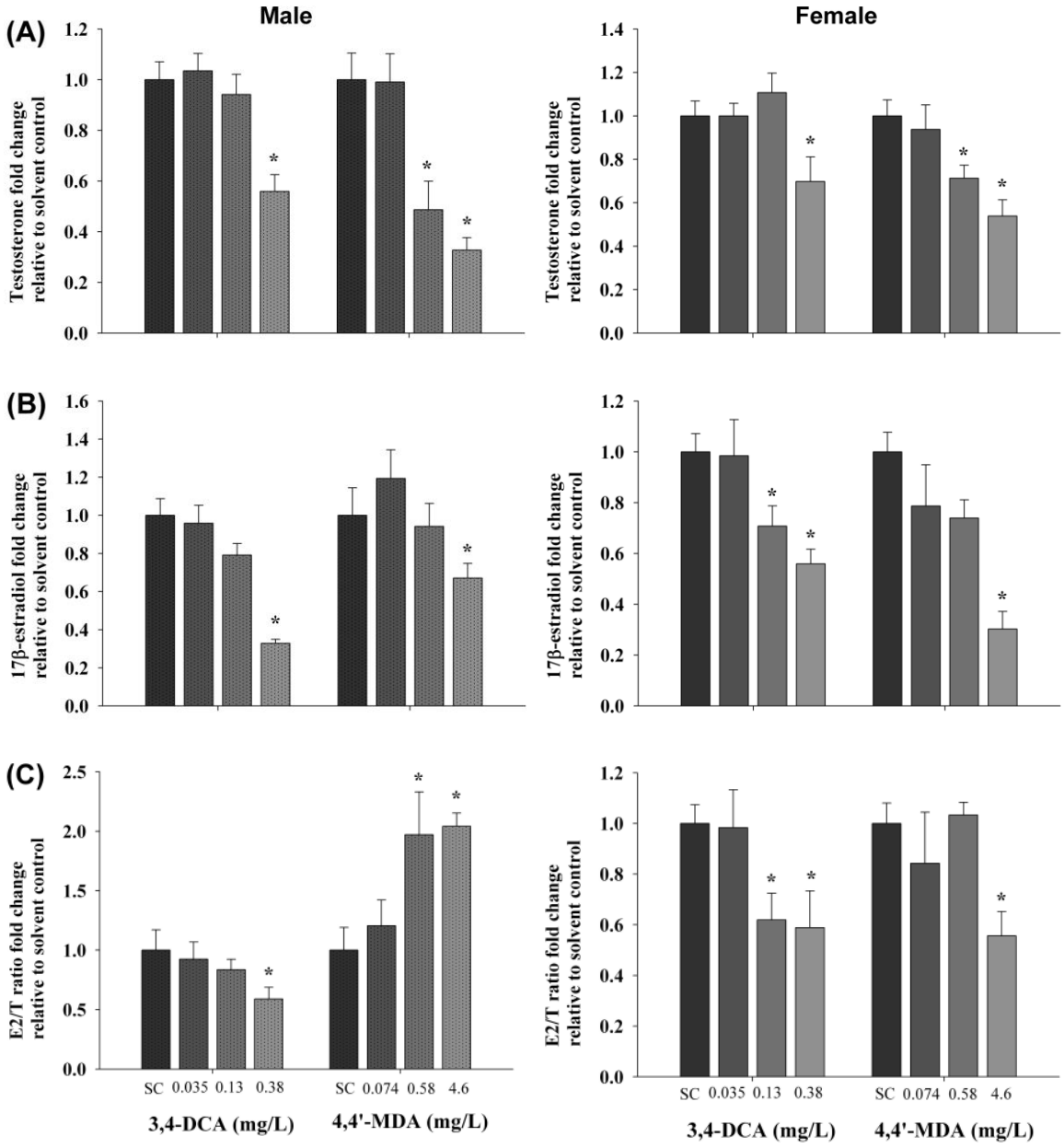


Figure 3.3. Plasma concentration changes of (A) Testosterone (T), (B) 17β-estradiol (E2), and (C) E2/T ratio measured in male and female zebrafish after 21 d of exposure to 3,4-DCA and 4,4'-MDA. Results are shown as mean±SD of four replicates for each concentration. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.005% DMSO).

3.3.4. Regulation of major steroidogenic genes in the HPG axis

Following the exposure to both aniline derivatives, significant regulatory changes were observed for major steroidogenic genes through the HPG axis both in the male and female zebrafish (Figure 3.4 and 3.5; for detailed information refer to Tables 3.3 and 3.4). The sex and organ-specific profiles of transcriptional change following exposrue to 3,4-DCA and 4,4'-MDA are summarized in Figures 3.4 and 3.5, respectively.

3.3.4.1. Brain

A significant upregulation of *gnrh2* gene was observed in both male and female fish brain at the highest exposure level of 4,4'-MDA. However, following exposure to 3,4-DCA, only female fish brain showed up-regulation pattern (Figure 3.4 and 3.5). The *gnrh3* gene showed a sex-dependent inverse direction of change in 4,4'-MDA exposed group, i.e., down-regulation in the male hypothalamus at all exposure levels, and up-regulation in the female at the highest exposure level (Figure 3.5). Following exposure to 3,4-DCA, it was up-regulated in the male fish at 0.38 mg/L (Figure 3.4).

In the fish pituitary, *gnrhr2* and *gnrhr4* genes up-regulated in the male fish, and only *gnrhr1* was up-regulated in female fish (Figure 3.4). The *gnrhr1* gene was up-regulated by 4,4'-MDA, whereas *gnrhr2* was down-regulated in the male fish and *gnrhr4* was up-regulated in the female fish, (Figure 3.5). A significant down-regulation of *fshβ* were observed in the male fish upon exposure to both 3,4-DCA and 4,4'-MDA. The *fshβ* gene was also down-regulated in the female following 3,4-DCA exposure, and *lhβ* gene was down-regulated by 4,4'-MDA exposure. The *cyp19b* gene was down-regulated in both the male and female fish upon the exposure to both test compounds. A significant down-regulation of *erα* was observed in the female by both compounds, while *erα*, *er2β*,

and *ar* genes were significantly up-regulated following exposure to 4,4'-MDA. The expression of *erα, er2β,* and *ar* genes were not affected in the male fish upon exposure to 3,4-DCA.

3.3.4.2. Gonad

Significant transcriptional changes were observed in the testis and ovaries generally at the highest concentration levels (Figures 3.4 and 3.5). After exposure to 3,4-DCA, the *fshr* gene was down-regulated only in the ovary while no changes in the transcription of *lhr* gene in the male or female fish 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 *hmgra* gene was down-regulated in the testis but no effect was observed in the ovary. A significant up-regulation was observed for the *hmgrb* gene in the ovary by 4,4'-MDA exposure. The *star* and *cyp19a* genes were significantly down-regulated in both male and female upon exposure to both the compounds. In addition, significant down-regulations were observed for *cyp11a* by 3,4-DCA and *17βhsd* by 4,4'-MDA exposure. A significant up-regulation of *3βhsd* and *cyp17* genes were also observed in both male and female fish gonads following exposure to 4,4'-MDA (Figure 3.5). The *ptgs2* gene, which is related to oocyte maturation and ovulation, was significantly down-regulated both in the male and female fish by 4,4'-MDA, and in the male fish by 3,4-DCA (Tables 3.3 and 3.4).

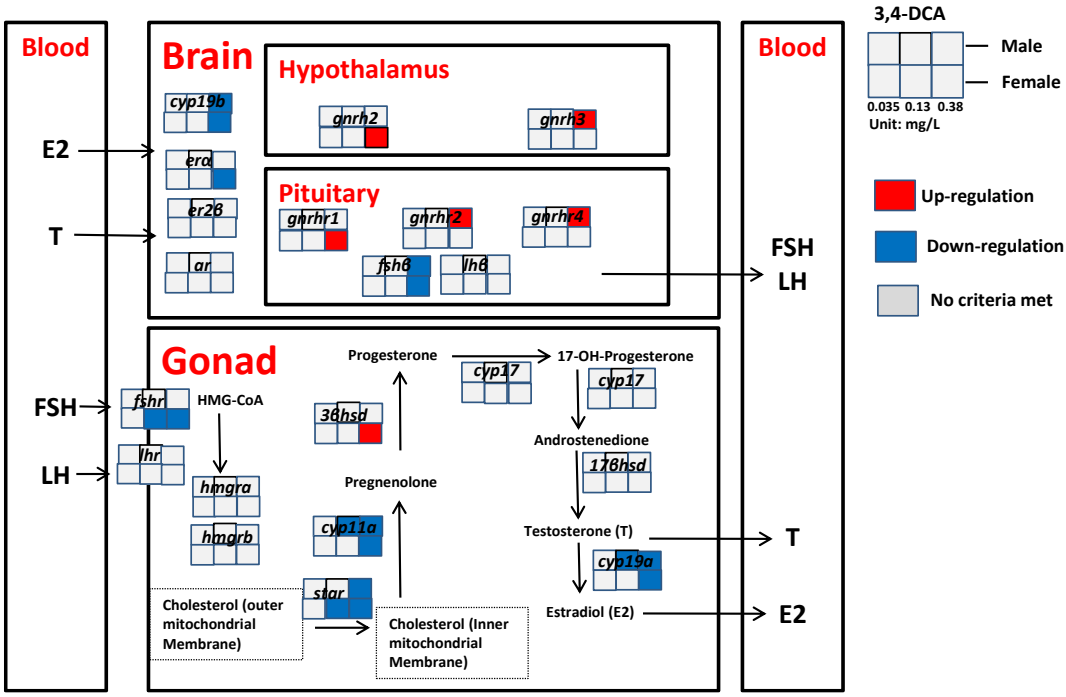


Figure 3.4. Effect on the major genes expression related to sex hormone regulation through the HPG axis of zebrafish upon exposure to 3,4-DCA. The order of exposure concentrations and order of sex are described by the legend on the upper right corner of the graph. The direction of regulation is shown in a different colour. Red: significantly up-regulate; blue: significantly down-regulate; grey: no statistically significant change.

Table 3.3. Effect on the mRNA expression of major sex hormone regulatory genes through the hypothalamus-pituitary-gonad axis in male and female zebrafish by 3,4-DCA.

Gene	Male			Female		
	0.035 mg/L	0.13 mg/L	0.38 mg/L	0.035 mg/L	0.13 mg/L	0.38 mg/L
Brain						
gnrh2	1.17±0.29	0.89±0.16	1.53±0.53	1.05±0.38	1.0±0.18	1.89±0.22*
gnrh3	1.09±0.25	0.98±0.12	1.56±0.24*	0.97±0.17	0.87±0.13	0.82±0.35
gnrhr1	1.01±0.22	1.1±0.22	0.94±0.18	0.99±0.12	1.11±0.13	1.72±0.13*
gnrhr2	0.96±0.29	0.95±0.16	1.57±0.16*	0.93±0.10	0.93±0.32	0.91±0.29
gnrhr4	0.94±0.21	1.1±0.14	1.64±0.12*	1.00±0.42	0.90±0.19	1.01±0.33
fshbβ	0.91±0.14	0.82±0.11	0.39±0.27*	1.09±0.17	0.90±0.12	0.57±0.16*
lhβ	0.95±0.32	0.98±0.13	1.09±0.38	1.0±0.46	0.93±0.18	0.78±0.15
cyp19b	0.89±0.22	0.96±0.29	0.47±0.12*	0.98±0.30	0.90±0.10	0.69±0.11*
erα	0.92±0.10	0.67±0.26	0.89±0.27	0.99±0.12	0.90±0.25	0.76±0.13*
er2β	1.0±0.47	0.87±0.32	0.96±0.13	1.09±0.26	1.37±0.49	1.12±0.36
ar	0.88±0.18	0.89±0.08	1.33±0.50	0.79±0.12	0.79±0.12	0.76±0.31
Gonad						
fshr	0.90±0.20	0.91±0.49	1.24±0.56	0.96±0.14	0.57±0.11*	0.33±0.14*
lhr	1.07±0.42	0.98±0.36	1.08±0.27	0.74±0.16	0.87±0.31	0.79±0.19
hmgra	1.01±0.28	1.10±0.32	0.74±0.26	1.0±0.22	0.95±0.09	0.89±0.15
hmgrb	0.83±0.15	0.91±0.55	1.08±0.35	0.97±0.12	1.03±0.33	1.06±0.18
star	1.04±0.09	0.87±0.27	0.71±0.11*	1.08±0.27	0.81±0.05*	0.62±0.20*
cyp11a	1.07±0.25	0.47±0.20*	0.33±0.15*	0.99±033	1.03±0.27	0.66±0.11*
3βhsd	0.65±0.16	0.58±0.29	0.44±0.09*	1.17±0.10	1.24±0.11	1.31±0.33*
cyp17	1.07±0.32	1.04±0.42	0.95±0.30	0.99±0.38	1.38±0.38	1.28±0.21
17βhsd	0.95±0.18	0.86±0.34	1.41±0.60	0.87±0.26	0.98±0.25	1.0±0.21
cyp19a	0.82±0.13	0.48±0.17*	0.63±0.17*	1.05±0.22	0.80±0.14	0.70±0.06*
ptgs2	1.06±0.32	0.84±0.40	1.58±0.30*	1.01±0.33	0.90±0.25	0.93±0.22

Note: The results are shown as mean ± standard deviation (n = 4 for each concentration for each sex). Gene expressions were expressed as fold change relative to solvent control. An asterisk indicates a significant difference between exposure groups and solvent control group ($p < 0.05$).

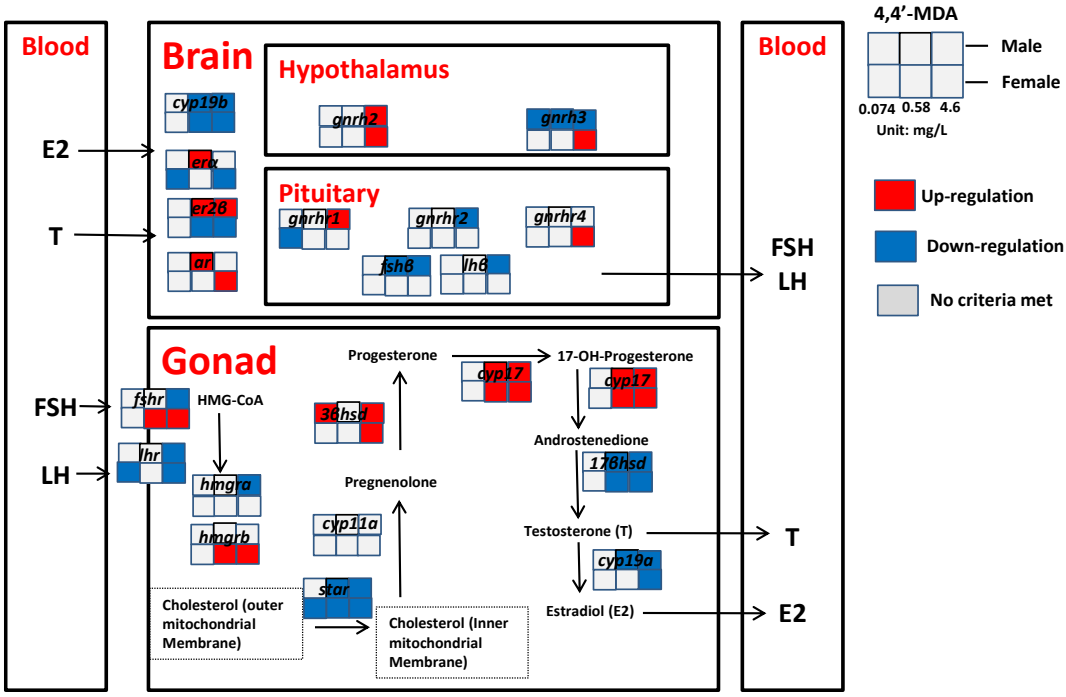


Figure 3.5. Effect on the major genes expression related to sex hormone regulation through the HPG axis of zebrafish upon exposure to 4,4'-MDA. The order of exposure concentrations and order of sex are described by the legend on the upper right corner of the graph. The direction of regulation is shown in a different colour. Red: significantly up-regulate; blue: significantly down-regulate; grey: no statistically significant change.

Table 3.4. Effect on the mRNA expression of major sex hormone regulatory genes through the hypothalamus-pituitary-gonad axis in male and female zebrafish by 4,4'-MDA.

Gene	Male			Female		
	0.074 mg/L	0.58 mg/L	4.6 mg/L	0.074 mg/L	0.58 mg/L	4.6 mg/L
Brain						
gnrh2	1.53±0.14	1.10±0.25	3.17±0.51*	0.97±0.15	1.01±0.19	2.16±0.51*
gnrh3	0.51±0.18*	0.48±0.07*	0.59±0.09*	0.93±0.29	0.89±0.17	1.39±0.27*
gnrhr1	1.31±0.32	1.21±0.09	1.36±0.12*	0.79±0.10*	0.73±0.21	0.80±0.14
gnrhr2	0.82±0.10	0.90±0.27	0.62±0.08*	1.36±0.51	1.03±0.42	1.51±0.81
gnrhr4	1.08±0.27	1.0±0.08	0.90±0.13	1.31±0.46	1.16±0.44	1.77±0.57*
fshbβ	0.85±0.15	0.65±0.15*	0.64±0.12*	1.18±0.22	1.06±0.38	1.22±0.26
lhβ	1.21±0.23	1.0±0.17	0.68±0.15*	1.42±0.42	1.13±0.36	1.35±0.32
cyp19b	1.24±0.09	0.80±0.13*	0.54±0.20*	0.87±0.28	0.75±0.13*	0.47±0.22*
erα	2.04±0.26	3.16±0.57*	1.56±0.27	0.67±0.14*	0.77±0.21	0.69±0.06*
er2β	1.94±0.17	3.71±1.17*	2.33±0.71*	0.63±0.19*	0.97±0.22	0.67±0.11*
ar	1.73±0.19	3.50±0.63*	2.12±0.59	0.81±0.17	1.12±0.39	1.46±0.38*
Gonad						
fshr	1.22±0.18	1.83±0.50	0.84±0.03*	0.78±0.24	1.44±0.15*	1.64±0.27*
lhr	1.019±0.10	0.98±0.28	0.43±0.16*	0.62±0.04*	1.16±0.27	0.76±0.02*
hmgra	1.13±0.12	1.0±0.15	0.64±0.06*	0.86±0.38	0.88±0.03	0.92±0.13
hmgrb	1.25±0.24	1.22±0.37	1.09±0.41	0.71±0.22	2.98±0.37*	3.04±0.66*
star	0.85±0.06	0.45±0.15*	0.29±0.03*	0.58±0.14*	0.51±0.06*	0.28±0.09*
cyp11a	1.49±0.27	1.60±0.74	1.60±0.23*	0.90±0.27	0.51±0.06*	0.79±0.41
3βhsd	2.07±0.55*	1.22±0.30	2.43±0.29*	0.63±0.30	1.71±0.24	3.35±1.35*
cyp17	1.63±0.33	2.81±1.0*	3.19±0.67*	0.56±0.22	2.33±0.33*	3.75±0.54*
17βhsd	0.66±0.23	0.41±0.13*	0.43±0.12*	0.76±0.14	0.62±0.16*	0.53±0.16*
cyp19a	1.17±0.10	0.66±0.16*	0.43±0.02*	0.93±0.28	0.72±0.15	0.63±0.12*
ptgs2	0.77±0.17	0.54±0.16*	0.47±0.06*	0.46±0.15*	0.80±0.04*	0.26±0.07*

Note: The results are shown as mean ± standard deviation (n = 4 for each concentration for each sex). Gene expressions were expressed as fold change relative to solvent control. An asterisk indicates a significant difference between exposure groups and solvent control group ($p < 0.05$).

3.4 Discussion

The observation of this study demonstrates that both 3,4-DCA and 4,4'-MDA disrupt the regulatory pathway of steroid hormone production, and also reduce reproduction. Plasma concentration of both testosterone (T) and 17β-estradiol (E2) level was significantly decreased by the tested compounds. The E2/T ratio was significantly decreased both in the male and female upon exposure to 3,4-DCA while sex-dependent difference of E2/T ratio was observed upon exposure to 4,4'-MDA, i.e., increased in male and decreased in the female. The ratio of E2/T is considered as a sensitive biomarker of sex hormone abnormality and is indicative of endocrine disruption (Ji et al., 2013; Orlando et al., 2004). Alterations of E2/T ratio in the fish following exposure to 3,4-DCA or 4,4'-MDA, along with reductions in egg number suggest that exposure to these two compounds could alter the sex hormones balance in fish, and as a consequence, cause adverse effects on reproduction of fish (Ji et al., 2013; Kwon et al., 2016; Shang et al., 2006). The changes in E2/T ratio observed in the male fish exposed to 3,4-DCA require careful interpretion because this observation is different from the results of a previous study (Bhuiyan et al., 2019). The reason for the discrepant results can be found from the difference in exposure levels and duration between the two studies. In addition, it is noteworthy that the extent of fold changes was small even though it was statistically significant. Moreover, in a separately conducted test to reproduce this observation, we could not find significant change of E2/T ratio even though the trend of decrease was similar (Appendix F).

The sex hormone changes observed in this study was supported by the regulatory

changes of the genes along the HPG axis in both sex fish. The up-regulation of *gnrh3* in the male and *gnrh2* in the female fish, respectively along with the up-regulation of *gnrhr2* in the male zebrafish and *gnrhr1* and *gnrhr4* in the female fish could suggest that 3,4-DCA could alter the concentration of gonadotropin hormones in the fish. Similarly, the up-regulation of *gnrh2* in both male and female fish, and sex-dependent regulatory chagnes of *gnrhr1*, *gnrh3*, and *gnrhr4* genes suggests that 4,4'-MDA could alter the concentration of gonadotropin hormones in the fish. Gonadotropin-releasing hormone (GnRH) and gonadotropins are major regulators of sex steroids. GnRH plays an important role in the reproduction of vertebrates (Okuzaoa et al., 2003; Tsutsumi and Webster, 2009). It regulates the production and release of gonadotropin hormones (GnRH). GnRH2 and GnRH3 are two types of GnRH with four different gonadotropin-releasing hormone receptors (GnRHR) (Tello et al., 2008). Therefore, sex hormones production affects GnRH through negative feedback. Hence, any alteration of GnRHs and GnRHRs can lead to changes in the sex hormone levels and therefore reproduction of the fish (García-Gómez, 2013).

Gonadotropin hormones released by the pituitary, act by binding to the receptors in the gonad. Two gonadotropin hormones, i.e., FSH and LH, bind with the receptors in the gonad (FSHR and LHR), and regulate steroidogenesis and gametogenesis (Kumar et al., 2001; Kwok et al., 2005). In the present study, *fshβ* gene was down-regulated in both male and female brain following the exposure to 3,4-DCA. In response to the regulatory change of *fshβ* gene in female fish, *fshr* gene was found down-regulated. However, *fshr* and *lhr* genes were not influenced in the male testis. Both spermatogenesis of male fish and estrogen production of female fish are regulated by FSH and LH (Schulz et al., 2010). The down-regulation of *fshβ* might lead to spermatogenesis disturbance of male

and estrogen imbalance of female fish. During the early stages of spermatogenesis, FSH has an important regulatory role, while during the later stages, maturation or spermiation are mainly regulated by LH (Ohta et al., 2007). Therefore, a potential interruption in spermatogenesis and maturation can be supported by the down-regulation of *fshβ, lhβ, fshr* and *lhr* genes in the male zebrafish.

Following 4,4'-MDA exposure, *fshβ, lhβ, fshr*, and *lhr* genes in male fish were down-regulated. The down-regulation of *fshr* and *lhr* genes in the testis of male fish at the highest exposure level might be a response to down-regulation of *fshβ* and *lhβ* genes. In the female fish, expression of *fshβ* and *lhβ* in the brain were not altered but *fshr* was up-regulated and *lhr* was down-regulated in the ovary after the exposure to 4,4'-MDA. In female fish, FSH primarily controls the vitellogenesis while LH controls the maturation of oocytes (Clelland et al., 2009). The up-regulation of *fshr* and down-regulation of *lhr* in the ovary suggest a receptor-mediated disruption of oocyte maturation and gametogenesis.

The effects of 3,4-DCA and 4,4'-MDA on the sex hormone regulatory pathway and reproduction impairment have not been reported to date. Testosterone decrease in the male and estradiol increase in the female Nile tilapia (*Oreochromis niloticus*) by 3,4-DCA were reported by Pereira et al. (2015; 2016). But the effects on regulation and reproduction of Nile tilapia have not been investigated. Decreased testosterone level observed in male zebrafish in the present study was comparable to the observations made in male tilapia fish (Pereira et al., 2015). However, we observed the opposite direction of response in the female zebrafish compared to those reported in the female Nile tilapia, i.e., E2 decrease in the present study vs increased E2 in Pereira et al. (2016). This difference may be due to the differences in species and exposure condition

(both male and female zebrafish exposed together in the present study).

Sex hormones can be used as integrative and functional endpoints that link to reproduction and at the same time transcriptional changes of steroidogenic genes (Ma et al., 2012). In the present study, significant decreases of T and E2 were accompanied by the down-regulation of *star*, *cyp11a* and *cyp19a* genes followed by 3,4-DCA, and *star*, *17βhsd* and *cyp19a* genes by 4,4'-MDA exposure. Significant down-regulation of major steroidogenic genes such as *star*, *cyp11a* and *17βhsd* supports decreased T concentrations. Down-regulation of *star*, *cyp11a* and *17βhsd* genes (Figures 3.4 and 3.5) may lead to decreased cholesterol uptake and eventually testosterone synthesis, which can explainreduced production of steroid hormones. The *star* gene plays an important role in which protein is responsible for the transport of cholesterol at the starting of the steroidogenic pathway into the mitochondrial membrane (Clewell et al., 2010). The enzyme hydroxyl methyl glutaryl CoA reductase (HMGR) is a rate-controlling enzyme of the mevalonate pathway that produces the cholesterol (Wang et al., 2015). The *hmgra* gene was down-regulated in the male fish, and *hmgrb* was up-regulated in the female fish. Therefore, in the male fish, both transportation and utilization of cholesterol are impaired, and in the female fish, only the cholesterol transportation is impaired following exposure to 4,4'-MDA. The enzyme encoded by the *17βhsd* gene is responsible for the conversion of androstenedione to testosterone. The enzyme, CYP19A catalyzes the conversion of T to E2 and regulates the aromatase activity which can affect the balance 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* gene transcriptions by both the tested compounds suggests that the aromatase activity was disrupted and therefore, E2 level decreases. In the fish brain, *cyp19b* gene expression is

a positive autoregulatory feedback of E2 concentration in the plasma (Collard et al., 2001). The down-regulation of *cyp19b* in male and female zebrafish brain following the exposure to 3,4-DCA and 4,4'-MDA may explain a lesser concentrations of E2. In a study with long-term exposure to tris (2-butoxyethyl) phosphate in male zebrafish, down-regulation of *cyp19a* and *cyp19b* genes in testis and brain, together with E2/ ratio increase were observed (Huang et al., 2019). Several fold up-regulation of *3β hsd* and *cyp17* genes were observed in both male and female fish gonads upon exposure to 4,4'-MDA. The product of *3β-hsd* is essential for the conversion of pregnenolone to progesterone, which in turn converts to 17α-OH-progesterone by the enzyme coded by *cyp17*. The up-regulation of *3β-hsd* and *cyp17* genes could be explained by the stimulation of basal synthesis of aldosterone and cortisol (Liu et al., 2010). Therefore, the concentration level of other steroid hormones measurement such as aldosterone and cortisol would be helpful to explain the consequences of the regulatoory changes of these two genes.

Regulatory changes of sex steroid hormone receptor genes showed an opposite direction in male and female brain, following exposure to 4,4'-MDA. In contrast, these genes were not influenced by 3,4-DCA, except down-regulation of *erα* gene in female fish. Down-regulation of *erα* and *er2β* observed in the female fish brain may explain lesser content of circulatory E2 level, and reduced E2/T ratio. But in the male fish, following 4,4'-MDA exposure, up-regulation of *erα* and *er2β* was observed, perhaps supporting estrogenic potentials of this derivative. Interestingly, a significant up-regulation of androgenic receptor gene was observed in both male and female while both both sex fish showed decreased T level, after exposure to 4,4'-MDA. Greater transcription of *ar* could be interpreted as a compensation against lesser production of T. Further

experimental validations are warranted for the present observations.

The enzyme cyclooxygenase (COX) catalyzes prostaglandin synthesis in follicles in teleosts, and is also responsible for maturation of oocytes and ovulation (Lister and Van Der Kraak, 2008; Sorbera et al., 2001). In zebrafish genome, one COX-1 gene (*ptgs1*) and two functional COX-2 genes (*ptgs2a* and *ptgs2b*) are recognized (Fujimoria et al., 2011). In our study, significant down-regulation of *ptgs2* was observed in both male and female fish gonads following exposure to 4,4'-MDA, and in the male fish by 3,4-DCA. The *ptgs2* gene expression is reported to be affected by chemical exposure and may influence reproduction. In the male fathead minnows, following exposure to ethinyl estradiol (EE2), *ptgs2* gene was up-regulated. In the female zebrafish, following exposure to (2-ethylhexyl)-phthalate (DEHP), this gene was down-regulated and also egg production was decreased (Garcia-Reyero et al., 2009; Carnevali et al., 2010).

In the present study, we report, for the first time, that the reproduction of fish and steroidogenic regulatory pathway could be affected by the exposure to 3,4-DCA and 4,4'-MDA. Transcriptional changes of several key steroidogenic genes may explain hormonal alteration of zebrafish (Figures 3.4 and 3.5). Significant reproduction decrease can be explained not only by sex hormone changes but also by significant down-regulation of *ptgs2* gene which suggest disruption of oocyte maturation and ovulation.

Consequences of long term exposure to 3,4-DCA and 4,4'-MDA in freshwater fish warrant further investigation.

Chapter 4 Summary and conclusions

To understand endocrine disrupting effect and toxicological consequences of major aniline derivatives, five anilines which include aniline and four of its derivatives, were chosen. The anilines chosed for the present study include aniline (ANL), 3,4-dichloroaniline (3,4-DCA), 1-naphthylamine (1-NPA), 4,4'-methylene dianiline (4,4'-MDA), and 4-chloroaniline (4-CA). First, an adrenal cell line, H295R cell line, was employed to screen the sex hormone disruption effects of the five chemicals. Sex hormones (T and E2) levels were measured and the expression level of four important steroidogenic genes, i.e., *StAR, CYP17, 3β-HSD,* and *CYP19A* were measured. 3,4-DCA, 1-NPA and 4,4'-MDA showed significant decrease in T level and increase of E2/T ratio. Among measured genes, *CYP17* was down-regulated by 3,4-DCA, 1-NPA, and 4,4'-MDA, and *CYP19A* was up-regulated by 4,4'-MDA. We could not find any hormonal and gene expression changes by ANL and 4-CA. Then, a zebrafish study was conducted to further screen the potential of endocrine disruption in the organism level. Male zebrafish were chosen because all three chemicals showed anti-androgenic effect in the cell line. In the male zebrafish, generally similar directions of the changes, i.e., decrease of T levels and increased E2/T ratios, were observed. Again, down-regulation of key steroidogenic genes such as *cyp17* or *3β-hsd*, but slight up-regulation of *cyp19A* gene were observed. The observations made on H295R cell and the male zebrafish demonstrate that all tested aniline derivatives could influence steroidogenesis and disrupt sex hormone balance which lead to reduced androgenicity.

In the second part, two aniline derivatives, i.e., 3,4-DCA and 4,4'-MDA, were chosen and their effects on fish reproduction were investigated, along with changes in

hormones and major gene transcriptions were investigated. Maiting pairs of zebrafish were exposed to each aniline derivative for 21 days, and were monitored for number of eggs producted daily. A significantly reduced egg numbers were observed together with significant decrease of sex hormones upon exposure to 3,4-DCA and 4,4'-MDA. The sex hormone change was supported by the regulatory changes of the genes through the HPG axis in both sex fish. Moreover, down-regulation of *ptgs2* gene was detected, suggesting disruption of oocyte maturation and ovulation were taken place by the exposure.

Our observations of this study demonstrate that some aniline derivatives i.e, 3,4-DCA, 1-NPA, and 4,4'-MDA have an adverse effects on the endocrine system which may influence steroidogenesis and eventually the reproduction of zebrafish. So, possible environmental risks of these chemicals warrant further evaluation. The information obtained from this study has an implication in ecological risk assessment of aniline and aniline derivatives.

The present study has some limitations. The exposure concentrations of this study are higher than environmental levels. While potential modes of endocrine disruption and their consequences could be understood, consequences due to exposure to environmentally relevant levels are not clear. Secondly, we did not measure 11-ketotestosterone (11-KT) in the fish, even though 11-KT has more important biological meaning. Finally, a hormonally active compound often shows low dose effect, but we did not include low concentrations in the experimental design. Consequences of long-term exposure to anilines at environmentally relevant concentrations should be investigated in the future.

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Appendices

Appendix A. Human biomonitoring data of anilines in the general population.

Chemical	Study	Type of population	Participants	Detection frequency	Urinary concentration (µg/L)	
					Median	range
ANL	Rifflemann et al., 1995		16		0.8	ND - 51
	Weisis & Angerer, 2002	General, Germany	160	100%	0.35	0.42-13
	Ward et al., 1996		26		2.9	NA
	el Bayoumy et al., 1986	General, USA	28		2.9	ND - 8.8
	Kütting et al., 2009	General Bavarian	1004	93.90%	3.1	0.05 - 384
4-CA	Kütting et al., 2009	General Bavarian	1004	38.20%	0.03	0.93
	Weiss and Angerer, 2002	General, Germany	20	90%	0.11	0.05-0.11
4,4'-MDA	Robert et al., 2007	General, French	120		0.05	0.10-0.87
3,4-DCA	Kütting et al., 2009	General Bavarian	1004	41.90%	0.03	0.42
	Turci et al., 2006	General, Italy	125	81.70%	0.08	0.01-6.19
	Weiss and Angerer, 2002	General, Germany	20	20%	<0.05	0.05-0.15
	Rubino et al., 2012	Agricultural worker	24		84 nmol	9.9–24,043 nmol
					9.25 nmol	3-67 nmol
						Blood concentration (fmole/mg Hb)
	Pasturelli et al., 2008	Agricultural worker				57-65
	Pasturelli et al., 2008					31 - 621

Appendix **B**. Aniline as a possible source of acetaminophen (paracetamol) in the urine of the general population.

Urinary concentration of Acetaminophen (no known occupational exposure to aniline or paracetamol use)				
Country	Participant type	Mean/ Median	Range (µg/L)	References
Danish	Children (6-11 yrs); urban	52170 µg/L	4.9-3037000	Nielsen et al., 2015
Danish	Children (6-11 yrs); rural	77200 µg/L	8.9-2546000	Nielsen et al., 2015
Danish	Mother; Urban	56800 µg/L	0-2257000	Nielsen et al., 2015
Danish	Mother; Urban	243 µg/L	2.79-4222	Nielsen et al., 2015
German	General	80 µg/L	8.4-2263	Dierkes et al., 2014
German	General	85.7 µg/L	8.7-22100	Modick et al., 2013

Appendix C. WST-1 cell proliferation assay

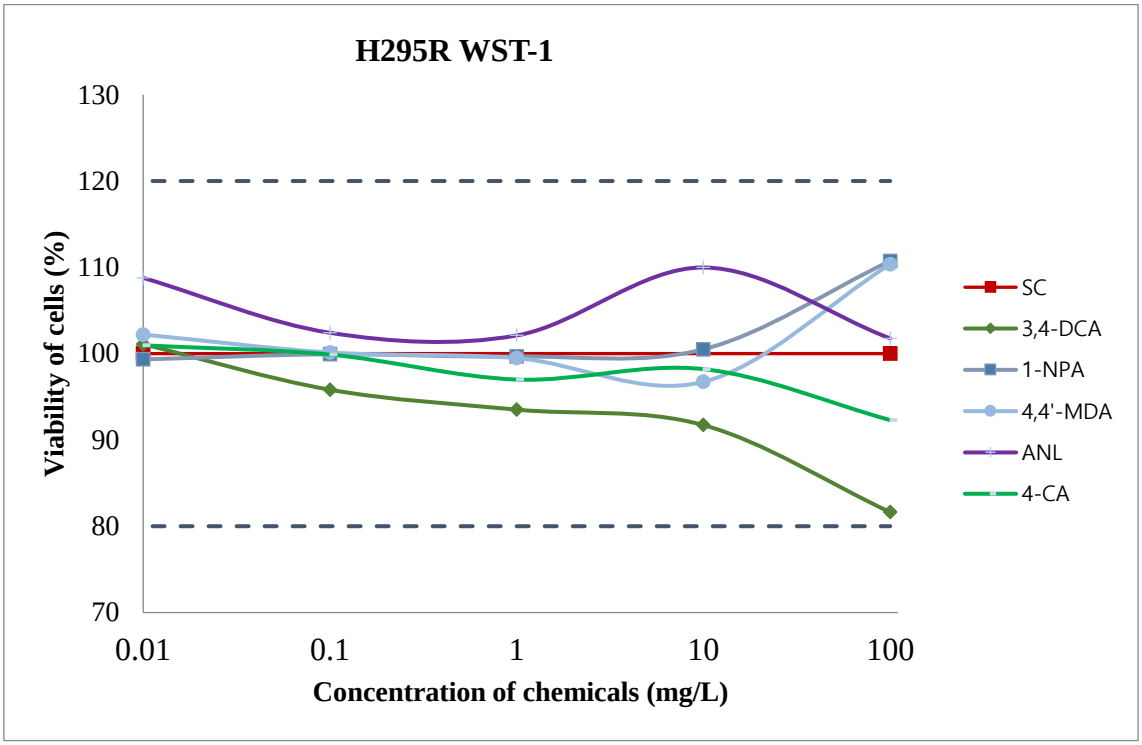

Figure. A1. Viability of the cells following the exposure to 3,4-DCA, 1-NPA, or 4,4'-MDA, in the WST-1 cell proliferation assay. Results are shown as mean ± SD of three replicates. SC represents solvent control (treated with 0.1% DMSO); Dotted lines represent +/- 20% deviation from the response of SC.

Appendix D. Hormonal changes by ANL and 4-CA in H295R cell.

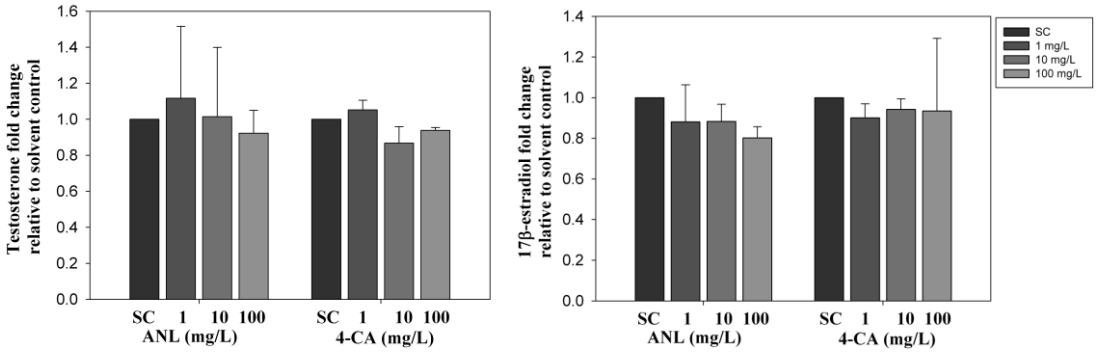

Figure. A2. Testosterone (T) and 17β-estradiol (E2) measured in the culture medium of H295R cells after 48 h exposure to ANL and 4-CA. Results are shown as mean ± SD of three independent experiments. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.1% DMSO).

Appendix E. mRNA expression level of *StAR*, *CYP17*, *3βHSD* and *CYP19A* gene in H295R cell.

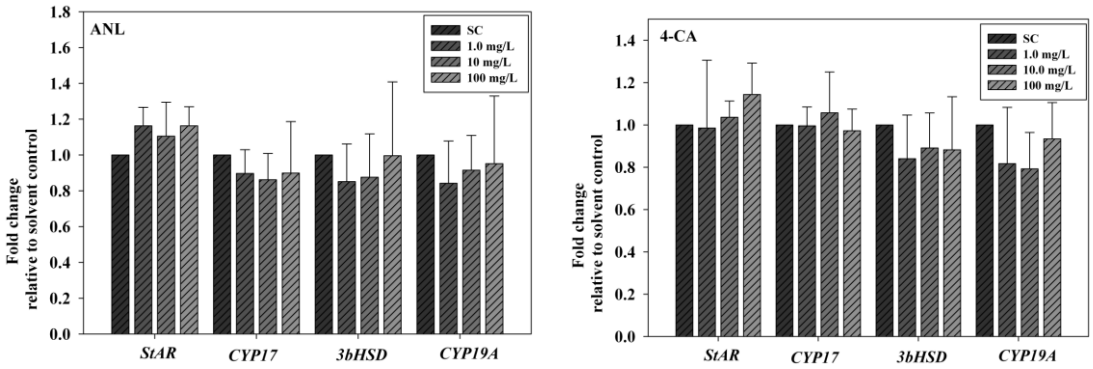

Figure. A3. mRNA expression level of *StAR*, *CYP17*, *3βHSD* and *CYP19A* gene in H295R cell after 48 h exposure to ANL and 4-CA. Results are shown as mean ± SD of three independent experiments. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.1% DMSO).

Appendix F. Hormonal changes of adult male zebrafish in reproducibility test upon exposure to 3,4-DCA for 21 days.

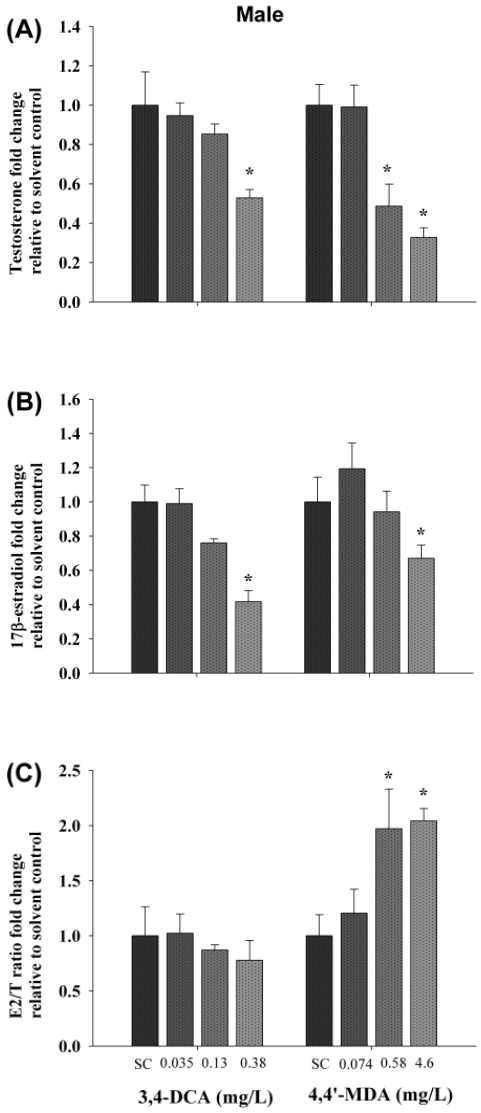

Figure. A4. Plasma concentration changes of Testosterone (T), 17β-estradiol (E2), and E2/T ratio measured in male zebrafish after 21 d of exposure to 3,4-DCA. Results are shown as mean±SD of four replicates for each concentration. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.005% DMSO).

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