

Effects of Octylphenol on Sexual Development and Reproduction in Zebrafish

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Abstract

Anthropogenic chemicals released into the aquatic environment can disrupt the normal function(s) of an organism's endocrine system and thereby adversely affect reproduction and development. This has led to the development of robust fish tests to detect and assess endocrine active chemicals. This thesis investigated developmental and reproductive effects of exposure to octylphenol (OP) in zebrafish.

Zebrafish were exposed to OP in a Fish Sexual Development Test (FSDT) to investigate effects on sexual development. The main endpoints were vitellogenin induction and gonad development, including sex ratios. Zebrafish were also exposed to OP in a Fish Full Life Cycle test (FFLC) and a Fish Short Term Reproduction Assay (FSTRA) to investigate effects on different reproductive processes; i.e. gonad development, sexual phenotype and reproductive performance.

Exposures to OP resulted in shifts in sex ratios, suppression in ovarian development, impairment in reproduction and reduction in growth, whereas no effects on VTG levels were observed. Conclusively, both the FSDT and FFLC test, but not the FSTRA, were sensitive tests for detection of endocrine-related effects of the weak estrogen OP. This thesis shows that OP, acting as a weak estrogen, has a negative impact on sexual development and maturation as well as reproduction in zebrafish.

Keywords: zebrafish, sex ratio, gonad maturation, vitellogenin, reproductive performance, octylphenol

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To my parents

To my husband and daughter

To the memory of my beloved grandmother

The Prophet Muhammad (peace be upon him) said: "One who treads a path in search of knowledge has his path to Paradise made easy by God..." - Riyadh us-Saleheen, 245

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Shaima A.M. Mahgiubi, Stefan Örn, Henrik Holbech, Gunnar Carlsson, Leif Norrgren, Gitte I Petersen. An inter-laboratory study to evaluate the effects of octylphenol on sexual development in zebrafish (*Danio rerio*) (manuscript).
- II Shaima A.M. Mahgiubi, Stefan Örn, Gunnar Carlsson, Leif Norrgren. Evaluation of octylphenol in a Fish Full Life Cycle test (FFLC) and a Fish Short Term Reproduction Assay (FSTRA) with zebrafish (*Danio rerio*) (manuscript).

Abbreviations

11-KT	11-ketotestosterone
AMH	Anti-mullerian hormone
ANOVA	Analysis of variance
dpf	Days post fertilization
dph	Days post hatch
E2	17 β -estradiol
EDCs	Endocrine disrupting chemicals
EE2	17 α -ethinylestradiol
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FET	Fish embryotoxicity test
FFLC	Fish full life-cycle
FSDT	Fish sexual development test
FSH	Follicle stimulating hormone
FSTRA	Fish short term reproduction assay
FTZ-F1	<i>Fushi Tarazu</i> factor-1
GnRH	Gonadotropin releasing hormone
GtH	Gonadotropin hormone
HE	Hematoxylin and eosin
hpf	Hours post fertilization
LH	Luteinizing hormone
LOD	Limit of detection
LOEC	Lowest observed effect concentration
LOQ	Limit of quantification
MeOH	Methanol
MIS	Maturation inducing steroid
MPF	Maturation promoting factor
NP	Nonylphenol
OECD	Organisation for economic cooperation and development

OP	Octylphenol
OPEs	Octylphenol ethoxylates
OSI	Ovarian somatic index
SC	Solvent control
Sox 9	SRY HMG box related gene 9
STWs	Sewage treatment works
VTG	Vitellogenin
WC	Water control

1 Introduction

1.1 General introduction

Many chemicals released by human activities can interfere with endogenous hormones which are important for sexual development and reproduction in vertebrates. Such interference may cause a number of developmental abnormalities and reproductive disturbances in human and wildlife (Toppari et al., 1996; Jobling et al., 1998; Olesen et al., 2007). These chemicals are identified as endocrine disrupting chemicals (EDCs). EDCs exert their action either by mimicking the behavior of endogenous sex hormones or blocking their actions, or by modulating their synthesis and metabolism (Kavlock et al., 1996). Many of these compounds end up in the aquatic environment. Therefore, fish and other aquatic species might be exposed to, and are particularly threatened by the action of EDCs.

In the last few decades, much effort has been spent by many international organizations such as the Organization for Economic Cooperation and Development (OECD) to develop reliable and robust test methods to identify hormonally active chemicals in aquatic environment. In this thesis, the potential effects of octylphenol on sexual development and reproduction in zebrafish (*Danio rerio*) were investigated using different fish tests.

1.2 Xenoestrogens

Compounds that behave as endogenous estrogens, called environmental estrogens or xenoestrogens, have been received the most attention. Xenoestrogens can exert their effects by binding with the estrogen receptor (ER) (Nimrod and Benson, 1996; Tyler et al., 1998). These include certain pharmaceuticals (e.g., 17 α -ethinylestradiol (EE2)), pesticides (e.g., lindane, endosulfan), and industrial chemicals (e.g., alkylphenols, bisphenol-A,

phthalates, polychlorinated biphenyls). Some of these compounds such as alkylphenols are persistent and can bioaccumulate. The main sources of xenoestrogens are municipal sewage effluent, industrial effluents and agricultural runoff. Xenoestrogens have been detected in sewage effluent, sludge, sediments, surface and ground waters at concentrations ranging from the ng/L level up to µg/L level (Ahel et al., 1994; Lee & Peart, 1995; Rudel et al., 1998; Larsson et al., 1999; Baronti et al., 2000; Kolpin et al., 2002; Sabik et al., 2003).

In fish, the period of gonadal development (sexual differentiation and maturation) has been shown to be very sensitive to xenoestrogens (Andersen et al., 2004; Holbech et al., 2006; Lin & Janz, 2006; Morthorst et al., 2010). A variety of developmental and reproductive abnormalities have been demonstrated in fish as a consequence to xenoestrogens exposure.

1.3 Effects of xenoestrogens in fish

It has been well documented that exposure to xenoestrogens during the sex differentiation period can cause feminization of male fish. Several examples of feminized male fish due to xenoestrogens have been reported in wild fish. For instance, a high frequency of intersex has been shown in wild roach (*Rutilus rutilus*) populations living in some English rivers that receive effluent from sewage treatment works (STWs) (Jobling et al., 1998). The incidence of intersex has been attributed to the presence of xenoestrogens in the sewage effluent. Intersex in wild roach has also been found in Scandinavian countries (Wiklund et al., 1996; Andersen *et al.*, 2001; Bjerregaard *et al.*, 2005).

One of the most reported effects of xenoestrogens is induction of vitellogenin. Elevation of vitellogenin levels related to xenoestrogens exposure has been shown in wild and caged fish living downstream of STWs (Purdom *et al.*, 1994; Harries *et al.*, 1996; Harries *et al.*, 1997; Lye *et al.*, 1997; Johnson *et al.*, 2008). In laboratory studies, increased vitellogenin concentrations have for example been documented in juvenile zebrafish following exposure to 17 α -ethinylestradiol, and to nonylphenol (Hill & Janz, 2003; Van den Belt et al., 2003). In other studies, female-biased sex ratio has been shown after exposure of juvenile fish to estrogenic compounds, such as 17 α -ethinylestradiol, and octylphenol (Knörr & Braunbeck, 2002; Örn et al., 2003).

It has been reported that developmental exposure to xenoestrogens resulted in delaying and suppression of gonadal development (gametogenesis). Delaying of sexual differentiation as expressed by increased the number of fish with undifferentiated gonads has for example been reported after

developmental exposure to 17 α -ethinylestradiol in zebrafish (Hill & Janz, 2003). Suppression of ovarian and testicular development has been shown in wild fish and in fish exposed to estrogens in laboratory studies (Papoulias et al., 1999; Dréze et al., 2000; Tanaka & Grizzle 2002; Weber et al., 2003; Kidd et al., 2007; Xu et al., 2008).

Another adverse effect is reduced reproductive capacity of fish (e.g., egg production, egg fertility and spawning success). This effect has been shown in wild fish and in fish exposed to estrogens in laboratory studies (Seki et al., 2003a; Van den Belt et al., 2001; Noaksson et al., 2005, Parrott & Blunt, 2005; Schäfers et al., 2007; Johnson et al., 2008). Xenoestrogens have also been shown to have the ability to bioaccumulate and transfer to offspring leading to toxic effects in the subsequent generation (Nice *et al.*, 2003).

Other examples of adverse effects of the xenoestrogens on fish are decreased gonadal weight, reduced ovarian maturity, increased number of atretic follicles and inhibited the production of seminal fluid (Van den Belt *et al.*, 2001; Van den Belt *et al.*, 2002; van der Ven *et al.*, 2003; Rasmussen & Korsgaard, 2004).

1.4 Xenoestrogen: Octylphenol

Octylphenol (OP) is used as an intermediate in the production of phenolic resins and octylphenol ethoxylates (OPEs). These chemicals are used in the manufacturing of textiles, paints, pesticides, detergents and cleaning agents. The main source for OP in the environment is microbial breakdown of OPEs during sewage treatment. OP can reach the aquatic environment via sewage treatment effluents. It has been detected in effluents of STW. For example, OP was found in effluent of STWs in Michigan with concentrations ranging from < LOD to 0.67 $\mu\text{g/L}$ (Snyder et al., 1999). It has also been found in STW effluents in Canada and Japan with concentrations ranging from 0.12 to 2.5 $\mu\text{g/L}$ and from 0.02 to 0.48 $\mu\text{g/L}$, respectively (Lee & Peart, 1995; Isobe et al., 2001). Concentrations of 0.15-39 $\mu\text{g OP/L}$ have been reported for untreated and treated wastewater (Rudel et al., 1998). OP has been measured in sediments and sludge at concentrations ranging from 9.2 to 12.1 $\mu\text{g/g}$ and from <0.005 to 0.91 $\mu\text{g/g}$, respectively (Lee & Peart, 1995). In the English Tees estuary, OP was measured at concentration of 13 $\mu\text{g/L}$ (Blackburn & Waldock et al., 1995). In Nordic countries, OP has been detected in various environmental compartments; for example, in sewage sludge, sediments, fresh and marine waters, fish, mussels, bird eggs and marine mammals (Hansen & Lassen, 2008).

OP has been shown to have significant endocrine disrupting effects on fish. Under laboratory conditions, OP has been reported to elevate vitellogenin levels (Jobling et al., 1996; Routledge et al., 1998; Gronen et al., 1999; Madsen et al., 2003; Pedersen et al., 2003), to impair testicular growth and spermatogenesis (Jobling et al., 1996; Gronen et al., 1999), to disrupt testicular morphology (Vázquez et al., 2009), to inhibit the production of seminal fluid (Rasmussen & Korsgaard, 2004) and to induce testis-ova in male fish (Gray et al., 1999a; Gronen et al., 1999). Moreover, OP has been documented to suppress oocyte development in female fish (Dumitrescu et al., 2010a). OP has been shown to bind with estrogen receptors in liver tissue (Andreassen & Korsgaard, 2000). In vitro studies, OP has been found to be the most potent estrogenic alkylphenol with potency approximately 10^{-3} to 10^{-7} relative to 17 β -estradiol (Jobling & sumpter, 1993; White et al., 1994; Arnold et al., 1996).

1.5 Gonad development in fish

A wide range of gonadal development patterns from hermaphroditism to gonochorism is present in fish. Many fish are gonochoristic, which may be differentiated or undifferentiated. In differentiated gonochoristic species such as medaka and common carp, undifferentiated gonad develops directly into an ovary or a testicle; while in undifferentiated gonochoristic species such as zebrafish, all individuals first develop gonads with ovarian tissue. There are also hermaphroditism species which may be simultaneous or sequential. In the simultaneous, both ovarian and testicular tissues are represented in the same organism at the same time and, in the sequential, organism reverse sex as adult from male to female (protandrous) or, from female to male (protogynous) (Devlin & Nagahama, 2002; Sandra & Norma, 2010). Gonad development includes sexual differentiation and maturation periods. During these periods the animal is most sensitive to the action of exogenous steroids.

In most fish, the undifferentiated gonads differentiate into ovaries or testis in the embryonic period (Sandra & Norma, 2010). Similar to other vertebrates, the main cell types of undifferentiated gonads in fish are primordial germ cells and somatic cells. For ovarian development, the somatic and germ cells begin to proliferate and differentiate to form follicles. Each follicle consists of the developing oocytes surrounded by two types of steroid-producing cells: inner granulosa and outer theca layer. The outer theca layer produces testosterone in response to gonadotropin action and the inner granulosa layer converts testosterone to estradiol. In the testis, the somatic cells differentiate to Sertoli cells and Leydig cells (interstitial cells). The Sertoli cells feed and support the

sperm cells during the stages of spermatogenesis: while Leydig cells produce the main male fish androgens, testosterone and 11-ketotestosterone.

1.6 Endocrine regulation of gonadal development in fish

Endogenous sex steroid hormones are the major regulators of gonadal sexual differentiation (Devlin & Nagahama, 2002, Sandra & Norma, 2010). However, the roles of endogenous hormones in sex differentiation are not fully understood. In several fish species, the presence of steroid-producing cells around the time of sexual differentiation suggests that sex steroids may play an important role in this process (Nakamura, 1985, 1993; Guiguen et al., 1999). Rougeot et al. (2007) reported significant increasing in the levels of sex steroids in Eurasian perch (*perca fluviatilis*) after onset of gonad differentiation. Moreover, it has been shown that treatment of fish with estrogenic or androgenic chemicals during the period of sex differentiation caused sex reversal (Du et al., 2003; Park et al., 2004). These results suggest that endogenous estrogens and androgens act as natural inducer of ovarian and testicular differentiation, respectively.

Gametogenesis (vitellogenesis and spermatogenesis) and final maturation in fish are also regulated by sex steroid hormones, which are produced in response to gonadotropin hormone (GTH) actions (Mylonas et al., 2010; Yousefian & Mousavi, 2011). The gonadotropin-releasing hormone (GnRH), which is produced from the brain, stimulates the release of gonadotropin hormones (GtHs) from the pituitary into blood stream. These gonadotropin hormones include follicle stimulating hormone (FSH) and luteinizing hormone (LH), which are in fish referred as to gonadotropin-I (GtH-I) and gonadotropin-II (GtH-II), respectively (Redding & Patiño, 1993; Nagahama, 1994). During vitellogenesis, sex steroid 17 β -estradiol (E2) stimulates the liver to produce and secrete vitellogenin into the blood stream, which is sequestered, processed and taken up into the oocyte through a receptor-mediated process enhanced by gonadotropin GtH-I (Swanson, 1994). GtH-II increases during the maturation stages and acts on ovarian follicle layer to produce maturation inducing steroid (MIS), which induces the formation of maturation-promoting factor (MPF) that further induces germinal vesicle breakdown and oocyte maturation (Nagahama & Yamashita, 2008). For testicular spermatogenesis and spermiation, GtH-I stimulates the production of 11-ketotestosterone (11-KT) from Leydig cells. This 11-KT is responsible for the full process of spermatogenesis, mediated also by growth factors secreted by the Sertoli cells (Mylonas et al., 2010; Yousefian & Mousavi, 2011). At spermiation, the

steroidogenic potency of GtH-II exceeds that of GtH-I leading to the production of maturation inducing steroid (MIS), which in turn activates specific enzymes that increase seminal plasma pH. As a consequence, spermatozoa capacitation and spermiation are induced (Mylonas et al., 2010; Swanson, 1994).

1.7 Zebrafish as a model organism

The zebrafish (*Danio rerio*) is a small tropical species, native to south-east Asia and belongs to the family of Cyprinidae (Laale et al., 1977; Spence et al., 2008). It has several advantages that make it a suitable model for developmental and reproductive studies. The fish has relatively short reproductive cycle (from 3-4 months), breeds throughout the year under appropriate laboratory conditions and produces a large quantity of eggs (approximately 50-200 eggs per day). Besides it is widely used as an experimental model in many international guidelines for risk assessments.

The genetic mechanism of sex determination in zebrafish is not clear. No sex chromosome has been identified in zebrafish (Schreeb et al., 1993; Pijnacker & Ferwerda, 1995; Traut & Winking, 2001; Wallace & Wallace, 2003). However, many autosomal genes such as the *Fushi Tarazu* factor-1 (FTZ-F1) gene, SRY HMG box related gene 9 (*Sox9*) and Anti-Mullerian hormone (AMH) have been proposed to contribute in the process of sex determination or differentiation in zebrafish (von Hofsten et al., 2005). Recently, germ cells have been found to play an essential role in female gonad development (Siegfried & Nusslein-Volhard, 2008). Environmental factors such as temperature (Uchida et al., 2004) have also been suggested as a factor influencing sex ratios in zebrafish.

Zebrafish is an undifferentiated gonochrostatic species i.e. all juvenile individuals begin to develop ovary-like tissues about 10-12 days after hatching regardless of their genetic sex. At the age of approximately 23-25 days, the sex differentiation period begins in which the ovary of genotypic female continues to grow into fully mature ovary, while in genotypic male, the ovary-like tissues degenerate and the sexual transformation of ovaries into testis takes place. At about 40 days post hatch, the process of sex reversal is completed and sexual maturation of gonads is generally finished at approximately 60 dph (Takahashi, 1977; Uchida et al., 2002; Örn et al., 2003). During sex differentiation period, zebrafish is highly sensitive to external factors involving exposure to xenoestrogens. Several studies have shown that exposure of zebrafish to single or binary mixtures of xenoestrogens during the period of

sexual differentiation can result in skewed sex ratio, elevated concentrations of vitellogenin and inhibited gametogenesis (Andersen et al., 2004; Holbeck et al., 2006; Lin and Janz, 2006; Morthorst et al., 2010). The zebrafish ovary is a bilobed, cystovarian type (Selman et al., 1993) and, develops asynchronously with oocytes at different growth stages. Oocyte development in zebrafish has been classified into following stages: primary growth stage, cortical alveolar stage, vitellogenesis, oocyte maturation and mature eggs (Selman et al., 1993).

2 Aims of the thesis

The overall aim was to apply available methods to investigate the potential estrogenic effects of octylphenol on sexual development and reproduction in zebrafish.

The specific aims were:

- To make an inter-laboratory comparison of the Fish Sexual Development Test (FSDT) for evaluation of the effects of octylphenol on sexual development in zebrafish.
- To assess the potential effects of OP on developmental and reproductive status of zebrafish in the Fish Full Life Cycle test (FFLC) and the Fish Short Term Reproduction Assay (FSTRA).

3 Material and Methods

A brief description of the material and methods used in this thesis is presented here. A more detailed description is given in Paper I and II.

3.1 Chemicals

Stock solutions of octylphenol (OP: 4(1, 1,3,3-tetra-methylbutyl)-phenol), were made in methanol (MeOH). The nominal test concentrations were 32, 100 and 200 µg/L OP (Paper I) or 1, 50 and 150 µg/L OP (Paper II).

3.2 Fish and husbandry

Adult zebrafish were bought from local importers, maintained in the laboratory at 26 ± 1.5 °C and on a light cycle of 12h light/12h dark. Fish were allowed to adapt to laboratory conditions for one month (Paper II) or two months (Paper I) prior to studies. Carbone filtered tap water and/or standardized water (Paper I & II) were used for embryo, juvenile and adult fish in the experiments. Fish fry were fed Liquifry 1 liquid suspension (Interpet, Dorking, Surry RH 4 3YX, England) and Sera micron (Heisenberg, Germany) powdered food for fry from 3-20 dph. Juvenile and adult fish were fed grinded Sera vipan flakes (Heisenberg, Germany), frozen fish food and newly hatched *artemia nauplii* (Ocean Nutrition Europe) three times daily.

3.3 Breeding of zebrafish

One day before egg collection, adult zebrafish were selected and transferred into 10-L glass aquaria having stainless steel mesh for breeding. Each breeding group included 10 fish (approximately 6 males and 4 females). About 2 hours after the onset of light in the morning, fertilized eggs were collected and

maintained in 1-2 L glass beakers containing carbon filtered tap water until 1 day post fertilization (dpf) (Paper I) or 10 days post hatch (dph) (Paper II). Thereafter, the fertilized eggs (Paper I) or the fry (Paper II) were transferred into the exposure aquaria.

3.4 Quantification of OP in water samples (Paper I)

Water samples (1 L) from each replicate were taken three times (Lab A) and six times (Lab B). The samples were stored at -20 °C until analyzed. Solid phase extraction (SPE) techniques followed by LC-MS analysis were conducted according to Rose et al. (2002) and Holbech et al. (2006) to determine the actual OP concentrations.

3.5 Fish assays for EDCs

3.5.1 Fish Sexual Development Test (FSDT) (Paper I)

The experimental protocol for FSDT used in this thesis is based upon an OECD test guideline (OECD, 2011). The test was carried out by two independent laboratories; Lab A (Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden) and Lab B (Department of Water and Environment, Danish Hydraulic Institute, Hørsholm, Denmark).

The test procedures are described in detail in Paper I. In general, juvenile zebrafish were exposed to OP from 1 dpf to 60 dph. The exposures were conducted under flow-through systems. The experimental setup included water control (WC) and exposure to 32, 100 and 200 µg/ L OP. The solvent control (SC) was included in the experiment of Lab A. Four replicate aquaria were used for each exposure group, as well as controls. Each aquarium contained 40 fish. At the day 60 post hatch exposure period was ended and fish were sacrificed.

3.5.2 Fish Full Life Cycle test (FFLC) (Paper II)

The test procedures are described in detail in Paper II. Briefly, fish were exposed between days 10-240 post hatch to nominal concentrations of OP at 1, 50 and 150 µg/L. The exposure was conducted under semi-static system and 50% of the water was changed every second day. Four replicate aquaria per OP concentration and six solvent controls were used. Each aquarium contained 20 fish. From 90 dph onward, fish were checked for the spawning. The reproductive performance of fish as fecundity, spawning success and fertilization success was monitored at the age of 180 dph for 21 consecutive

days and, embryo development in offspring was screened until 144 hours post fertilization (hpf). Various developmental endpoints were studied in offspring and summarized in Table 1. At 240 dph the exposure was stopped and fish were sampled for VTG analysis and gonad histopathology evaluation.

Table 1. *Developmental endpoints studied at 24, 48 and 144 dpf in zebrafish embryo*

Endpoints	Description	24hpf	48hpf	144hpf
<i>Categorical endpoints</i>				
Coagulation	Embryo is coagulated, yes/no	+	+	+
Heart beats	Embryo has visible heart beats, yes/no		+	
Tail development	Embryo has short or curved tail, yes/no	+	+	+
Circulation	Embryo has visible blood flow in tail artery, yes/no		+	
Pigmentation	Embryo shows clear pigmentation in eyes and on body surface, yes/no		+	
Edema	Edema is present, yes/no		+	+
Hatching success	Embryo is hatched, yes/no			+
<i>Continuous endpoints</i>				
Movements	Number of movements/min.	+		
Heart rate	Time for 30 heart beats is recorded and present as number of beats/minute		+	

3.5.3 Fish Short Term Reproduction Assay (FSTRA) (Paper II)

The experimental protocol for FSTRA used in this thesis is based on an OECD test guideline (OECD, 2009). Adult male and female zebrafish were held together in spawning cages within 10-L aquaria. Each aquarium contained eight fish (approximately 5 males and 3 females). The fish were exposed to OP (1, 50, 150 µg/L) for 21 days. The experimental period consisted of a 7-day pre-exposure period followed by a 21-day exposure period. During both pre- and exposure periods, fecundity, spawning success and fertilization success were monitored daily. After 21 days of exposure all fish were sampled for VTG analysis and gonad histopathology evaluation.

3.6 Fish sampling

Fish sampling was performed at termination of tests. Fish were anaesthetized with MS-222 (Pharmaq, UK), measured for body weight and length, and decapitated. The condition factor for each individual fish was calculated ($\text{weight (g)/ length (cm)}^3 \times 100$) (Paper I). Head and tail parts from all fish

(Paper I) or from adult male fish (2-5 individuals per replicate aquaria) (Paper II) were removed, frozen immediately in liquid nitrogen and stored at -80 C° until quantification of VTG. The mid-body of each fish was fixed for histological examination.

3.7 Sex determination and gonad evaluation

Fixed mid-body of each fish was processed to determine the sex of each fish and, to evaluate the maturity of the gonads. The tissues were dehydrated in graded serial of ethanol and embedded in paraffin blocks. Longitudinal sections were cut at 5µm thickness, collected onto glass slides, stained with hematoxylin and eosin (HE), and examined under light microscope.

Based on histological appearance, the gonadal sexes of the fish were classified into four groups: testis, ovary, intersex or undifferentiated. Ovarian maturity was determined according to the stages defined by Selman et al. (1993): primary growth stage, cortical alveolus stage and vitellogenic stage. The histological sign for maturing ovaries was the presence of cortical alveolus oocytes (Paper I). The ovarian maturity was also evaluated by counting the number of different oocyte maturation stages per microscopic field and calculating the proportion of each cell type as well as atretic follicles (Paper II). Testicular maturity was assessed according to the presence of spermatozoa (Paper I & II) and amount of spermatozoa in sperm duct (Kinnberg et al., 2007) (Paper I). The testicular maturity was also assessed by counting the number of spermatid cysts per microscopic field (Paper II).

3.8 Vitellogenin analysis

Vitellogenin concentrations were quantified in male, female and undifferentiated fish (Paper I) or in adult male fish (Paper II). The analysis was performed using a direct non-competitive sandwich ELISA, based on polyclonal affinity purified antibodies against zebrafish lipovitellin as described by Holbech et al, (2001).

3.9 Statistics

Kruskal-Wallis test followed by the Mann-Whitney *U* test with Bonferroni adjustment was used to analyze data on VTG concentrations (Paper I), body length (Paper I), and ovarian maturation stages (Paper II). Fisher's Exact Test followed by Bonferroni adjustment of p-values was used to test differences between control groups and each exposed group in sex ratio (Paper I) and in all

categorical endpoints studied at 24, 48 and 144 hpf in zebrafish embryo (Paper II). All other data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnet's post hoc test (Paper I&II). Statistical analysis of data was calculated using MINITAB release 15. The significant level was set at 95% ($P < 0.05$).

4 Results

4.1 Measured OP concentrations in water samples (Paper I)

Mean measured concentrations of OP were lower than the nominal concentrations in both experiments (Table 2). The concentration of OP in water of control tanks was less than the limit of quantification (LOQ) at both labs. Low $\mu\text{g/L}$ concentrations of OP were detected in solvent group tanks at Lab A.

Table 2. Nominal and measured (mean \pm S.D) concentrations of OP in water samples.

Nominal Conc.	Lab A		Lab B	
	Mean Measured	% nominal	Mean Measured	% nominal
WC	<LOQ	-	<LOQ	-
SC	3.3 \pm 8.0	-	-	-
32 $\mu\text{g/l}$	5.7 \pm 5.4	17.8	13.8 \pm 8.0	43
100 $\mu\text{g/l}$	17.6 \pm 19.4	17.6	40.6 \pm 8.0	40.6
200 $\mu\text{g/l}$	42.5 \pm 8.0	21.25	73.1 \pm 8.0	36.6

< LOQ = below limit of quantification.

4.2 Sex ratio

Partial life-cycle exposure of zebrafish to OP altered the sex ratio (Paper I). A decrease in proportion of males and an increase in proportion of undifferentiated fish were shown after exposure to OP at concentrations $\geq 100 \mu\text{g/L}$ and $\geq 32 \mu\text{g/L}$ at Lab A and B, respectively. More females were observed following exposure to all concentrations of OP at Lab B, but not at Lab A. In total the occurrence of intersex fish was very low, i.e. 0.64% at Lab A and 1% at Lab B. No effects were detected on adult sex ratio in zebrafish after life-long exposure to OP (1-150 $\mu\text{g/L}$) (Paper II).

4.3 Gonad Maturation

Partial life-cycle exposure of zebrafish to OP caused a concentration-related suppression in ovarian maturity at nominal concentrations $\geq 32 \mu\text{g/L}$ and $\geq 100 \mu\text{g/L}$ at Lab A and B, respectively (Paper I). In Paper II, full life-cycle exposure to OP at $150 \mu\text{g/L}$ impaired ovarian maturation, as shown by increased proportion of immature oocytes (perinuclear oocytes) in proportion to the other stages present. Concentration-related increases in follicular atresia were also seen, albeit statistically not significant. The histological examination of ovaries in adult zebrafish exposed to OP (1, 50, $150 \mu\text{g/L}$) for 21 days did not reveal any differences in proportions of oocytes at different maturation stages (Paper II). Testicular maturity was not affected by exposure to OP (Paper I & II).

4.4 Vitellogenin

Vitellogenin levels in males, females and undifferentiated fish exposed to OP ($32\text{-}200 \mu\text{g/L}$) were not significantly different from those in the controls for any exposure concentration at both Labs (Paper I). No vitellogenin induction was detected in males after full life-cycle exposure to 1, 50 and $150 \mu\text{g/L}$ OP (Paper II). Similarly, no changes in VTG levels were observed in male zebrafish exposed for 21 days to 1, 50 and $150 \mu\text{g/L}$ OP (Paper II).

4.5 Growth Parameters

Partial life-cycle exposure of juvenile zebrafish to OP (32, 100, $200 \mu\text{g/L}$) caused significant decrease in body weight, but not length, of females at concentrations $\geq 32 \mu\text{g/L}$ and $\geq 100 \mu\text{g/L}$ at Lab A and B, respectively (Paper I). The Condition factor was significantly higher in females exposed to $200 \mu\text{g/L}$ OP at Lab B (Paper I). In Paper II, full life-cycle exposure of zebrafish to OP ($150 \mu\text{g/L}$) caused significant decrease in body weight of females, but not length. No significant effects in body weight or length of males were recorded after exposure to OP (Paper I & II). Adult exposure to OP for 21 days did not result in adverse effects on growth of both males and females (Paper II).

4.6 Reproductive parameters (Paper II)

Full life-cycle exposure of zebrafish to OP at $150 \mu\text{g/L}$ reduced fertilization success. The fecundity and spawning success, although statistically not significant, were also reduced at the same OP exposure concentration. Adult exposure to OP for 21 days had no effects on reproductive parameters.

4.7 Embryo development (Paper II)

Offspring from the fish that were exposed during 10-240 dph to the highest OP concentration showed increased mortality. No other effects were observed in the offspring.

5 Discussion

This thesis investigated developmental and reproductive effects of exposure to octylphenol (OP) in zebrafish using three different tests. In the FSDT, the measured concentrations of OP in the water were much below the nominal concentrations at both labs. Declines in OP concentrations have been observed in other studies and have been attributed to be due to uptake by fish, adsorption to the glass, microbial degradation and photolysis (Bangsgaard et al., 2006; Jespersen et al., 2010; Vázquez et al., 2009). However, there were variations between Labs (Lab A and Lab B) with regards to the mean measured OP concentrations and the lowest observed effect concentrations (LOECs) of OP for sex ratio, ovarian development and female body weight. The mean measured OP concentrations at Lab B were approximately two times higher than the concentrations measured at Lab A. This variation is probably due to difference in water renewals in the flow-through test systems. The LOECs for OP on the sex ratio were 17.6 µg/L and 13.8 µg/L at Lab A and Lab B respectively. Additionally, the LOECs for OP on the ovarian development and female body weight were 5.7 µg/L and 40.6 µg/L at Lab A and Lab B respectively. Factors that may have caused this inter-laboratory variation in results are strain differences, fish genetics, stock sensitivity, and amount of food.

Sex ratio has been shown to be a sensitive endpoint in testing of EDCs with different mode of action (e.g., estrogens, androgens and aromatase inhibitors) (Lange et al., 2001; Örn et al., 2003; Holbech et al., 2006; Panter et al., 2006; Kinnberg et al., 2007; Morthorst et al., 2010). Partial life-cycle exposure of zebrafish to OP changed sex ratio (Paper I). Three different observations were made: there were either more females with consequently less males, or more females along with more undifferentiated fish, or less males together more undifferentiated fish. The zebrafish is an undifferentiated gonochorist and both sexes have ovary-like tissues before sexual differentiation. The transformation

of ovary-like tissues into either ovary or testis depends on the levels of endogenous estrogens. Therefore, exogenous estrogens may counteract with this transformation. Our findings suggest that OP delays male sex differentiation or causes feminization of males. Exposure of fish to weak or potent estrogenic compounds has in several studies shown to alter sex ratio. Lin and Janz (2006) documented that exposure of juvenile zebrafish to 10 µg/L nonylphenol (NP) skewed sex ratios toward females, with complete sex reversal occurring after exposure to 100 µg/L. Further, Hill and Janz (2003) reported a decreased number of phenotypic males after developmental exposure of zebrafish to nominal concentrations of 100 µg/L NP and 10 ng/L EE2, with an increased number of undifferentiated fish in the groups exposed to EE2. In the present study, the occurrence of intersex fish was very low. Several studies have reported low occurrence of intersex in zebrafish after exposure to estrogenic compounds (Nash et al., 2004; Fenske et al., 2005). Higher incidence of intersex has been observed in other fish species after exposure to estrogens (Gray et al., 1999a; Lange et al., 2001). Our results suggest that zebrafish may be less sensitive than other fish species in terms of intersex development. No sex ratio response was observed in adult zebrafish after lifelong exposure to OP (1-150 µg/L) (Paper II). Measurement of OP concentrations in the FSDT test showed rather low actual concentration even though water was renewed three times daily in the flow-through system. In this long-term exposure water was renewed with 50 % every second day, why actual concentrations probably are even lower than that of the FSDT test, and thereby the weak response in the study. In contrast to our finding, female-biased sex ratios were observed in Japanese medaka continuously exposed to OP from fertilization to maturity (Knörr & Brubeck, 2002).

Histological analysis of gonad maturation is a valuable endpoint in risk assessment of EDCs. Ovarian maturation was the most sensitive endpoint to OP in the present studies. Partial life-cycle exposure of zebrafish to OP caused suppression in ovarian maturity at both Labs (Paper I). In Paper II, full life-cycle exposure to highest concentration of OP also impaired ovarian maturation. The suppression in ovarian development observed in both studies proposes that OP inhibits female gametogenesis. Many studies have shown inhibition of ovarian development in fish following exposure to alkylphenols (Tanaka & Grizzle et al., 2002; Weber 2003; Dumitrescu et al., 2010a). Concentration-related increases in follicular atresia, although not significant, were seen after full life cycle exposure to OP. A possible explanation might be that the OP affects the final maturation of oocytes by inhibiting maturation inducing steroid (MIS). In a previous *in vitro* study it was shown that treatment of zebrafish oocyte follicles with exogenous E2 caused a significant inhibition

of MIS (Pang & Thomas, 2009). Testicular maturity was not affected by exposure to OP (Paper I & II). Inhibition of testis development has been shown in fish following exposure to estrogens, such as 17 α -ethinylestradiol and nonylphenol (Popoulias et al., 1999; Dréze et al., 2000; Weber et al., 2003).

Vitellogenin (VTG) is an egg yolk protein, synthesized in the liver of mature oviparous female vertebrates such as fish under the control of estrogens. VTG is not normally found in males and juvenile fish, but it can be induced after exogenous estrogen exposure (Sumpter & Jobling, 1995; Folmar et al., 1996; Jobling et al., 1996; Tyler et al., 1996). VTG has thus been used as a biomarker for detection risks and effects of EDCs in different fish species. In the present studies, VTG concentrations were not significantly different from those in the controls for any exposure concentration at both Labs (Paper I). No effects on VTG concentrations were seen in males exposed to OP from 10 to 240 dph (Paper II). Similarly, No VTG was detected in adult males exposed for 21 days to OP (Paper II). In contrast to our findings, estrogenic effect of alkylphenols in term of vitellogenin induction has previously been shown in fish. For example, exposure of juvenile zebrafish to closely related nonylphenol (NP) from 2-60 dph resulted in induction of VTG starting at a nominal concentration of 30 $\mu\text{g/L}$ (Hill & Janz, 2003). Further, life cycle exposure of medaka to the weak estrogen 4-*tert*-pentylphenol elevated VTG levels in adult males at concentrations $\geq 51.1 \mu\text{g/L}$ (Seki et al., 2003a). Also Gronen et al. (1999) reported induction of VTG in adult male medaka exposed for 21 days to OP at concentrations of $\geq 20 \mu\text{g/L}$. The non-significant effects of OP on levels of VTG observed in the present work indicate that VTG induction is not always sensitive endpoint to compounds with weak estrogen potency.

Partial life-cycle exposure of juvenile zebrafish to OP (32, 100, 200 $\mu\text{g/L}$) caused significant decreases in body weight of females at both Labs (Paper I). The reduction in female body weight was also observed after full life-cycle exposure to 150 $\mu\text{g/L}$ OP. Effects on ovarian development could be an explanation for a reduction of female body weight observed in the present studies since most females had immature ovaries. Reductions in body weights have previously been reported in both zebrafish (Dumitrescu et al., 2010b), Japanese medaka (Knörr & Braunbeck, 2002) and in rainbow trout (Ashfield et al., 1998) following exposure to OP.

For determination of the potential impacts of EDCs on the population level, effects on reproductive performance can be studied. Full life-cycle exposure of zebrafish to OP caused significant reduction in fertilization success at the highest OP concentration, 150 $\mu\text{g/L}$. The fecundity and spawning success were also reduced at the same OP concentration, albeit not significant. The reproductive disorders observed in this study probably due to impaired ovarian

development. Reduced fertility has been shown in fish following life-long exposure to potent or weak estrogenic substances (Seki et al., 2003a; Schäfers et al., 2007). Adult exposure to OP for 21 days had no effects on reproductive parameters. Van den Belt et al. (2001) also did not show effects on reproductive parameters in adult zebrafish after exposure for 21 days to OP (12.5-100 µg/L). In the FFLC test a significant increase in mortality at 144 hpf in offspring from parental fish exposed to the highest OP concentration was recorded. The reason for this might be disturbances in the development and maturation of the eggs because of general toxicity in females, i.e. bad egg quality due to e.g. nutritional factors. However, it might also indicate possible mechanisms for maternal transfer of OP from the females directly into the eggs and thereby affecting the embryos during early development. Exposure of parental medaka to OP has been shown to cause developmental problems in embryos (Gray et al., 1999b). Further, nonylphenol has been shown to have the ability to bioaccumulate and transfer to offspring leading to toxic effects in subsequent generation (Nice *et al.*, 2003).

Identifying chemicals with endocrine activity requires screening and testing by use of validated methods. The Fish Sexual Development Test (FSDT) is intended to assess early life-stage effects and possible adverse outcomes of EDCs with different mode of action (e.g., estrogens, anti-estrogens, androgens and aromatase inhibitors). During recent years the FSDT has been validated and the assay was recently accepted as a standard OECD guideline for detection and evaluation of the effects of EDCs (Holbech et al., 2006; Kinnberg et al., 2007; OECD, 2011). The FSDT includes mode of action endpoints sex ratio and VTG. Therefore, it is used as a definitive test for hazard and risk assessment. In this test, fish are exposed to the chemical of concern during the period of sexual differentiation, which is the most sensitive period to be affected by EDCs. In the present FSDTs, the LOECs of OP for studied endpoints were 5.7 (Lab A) and 13.8 µg/L (Lab B). This is comparable with that reported for medaka exposed to OP from fertilization to 60 dph (11.4 µg/L) (Seki et al., 2003b). The Fish Full Life-Cycle test (FFLC) is needed in ecotoxicological assessment of endocrine active substances. The test is being developed to evaluate the potential effects of EDCs at population level. It is a comprehensive chronic test in which fish are exposed to the test substance from early life stage to adulthood stage. This test is valuable since it can be conducted with a variety of developmental and reproductive endpoints. In the present FFLC test, negative effects on growth, ovarian maturation, reproduction as well as offspring quality in zebrafish were only seen at the nominal concentration of 150 µg/L. This concentration is higher than those reported in other long term exposure testes. The LOECs documented for

medaka after long term exposure to OP were 10µg/L and 2 µg/L (Gray et al., 1999b; Knörr & Brubeck, 2002). The reason for differences in LOECs between the studies may be due to differences in the actual concentrations. The FSTRA is designed and validated for screening of EDCs. In this test, sexually mature male and female fish are exposed to the test substance for a short period; i.e. 21 days. It includes measurements of both biomarkers (e.g. VTG) and apical endpoints (e.g., fecundity). The present FSTRA was not sensitive enough to detect the effects of OP on the studied endpoints at these nominal concentrations. This disagrees with Gronen et al. (1999) who showed impacts on VTG levels and reproduction in adult medaka exposed to OP (20-230 µg/L) for 21 days.

The results observed in the FSDT, FFLC test as well as FSTRA suggest that in zebrafish the most responsive period for exposure to estrogens is the period of sexual differentiation, while exposure during adulthood period is less sensitive. However, the results also suggest that the exposure periods from 1dpf to 60 dph and from 10 to 240 dph are suitable to evaluate the potential effects of estrogenic compounds on sexual development and reproduction in zebrafish.

6 Conclusion

- Zebrafish is sensitive to weak estrogen compounds at environmental relevant concentrations and ovary maturation and development of phenotypic sex are the most sensitive end-points.
- The period of sex differentiation is appropriate for studies on gonadal development and reproductive toxicity in zebrafish whereas the adulthood period is less vulnerable.
- FSTRA, FSDT and FFCL test are all valuable assays and depending on the objective with a study and properties of the chemical the assays can be utilized one by one or in combination.

7 Future research

During the last decade, the zebrafish has passed through a rapid period of development as a species from many perspectives for instance in basic research, as a model for regulatory work and risk assessment. Genomics and other -omics are examples of methodologies, which have been developed. In order to further develop the zebrafish as a model species for evaluation of EDCs it is necessary to learn more about normal and disturbed gonadogenesis both in males and females. Also, colouration, fin size, the presence and length of the gonophore are examples of apical sex characteristics, which can be assessed to follow a process of feminization or masculinization. Another area, which should be evaluated is reproductive behaviour such as aggressiveness, attraction, escape and swimming patterns. Existing assays could be further developed for instance the Fish Embryotoxicity Test (FET) could be more useful by modifications such as dechorination, nanoinjection of substances into the yolk and introduction of metabolic systems. The most complex test the FFLC test is especially demanding and it is a great challenge to develop and optimize the FFLC test for studies of transgenerational effects.

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