Zebrafish (*Danio rerio*) and Japanese Medaka (*Oryzias latipes*) as Model Species for Evaluation of Endocrine Disrupting Chemicals

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The present thesis is a partial fulfilment of the requirements for an Master of Science Degree in Veterinary Medicine for International Students at the Swedish University of Agricultural Sciences (SLU), in the field of Pathology.

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To my Parents and God

Life is beautiful

Be civil to all; sociable to many; familiar with few; friend to one; enemy to none.

Benjamin Franklin

Contents

ABBREVIATIONS

DDE	1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene
DDT	1,1,1,-trichloro-2,2-bis(4-chlorophenyl)ethane
DNA	deoxyribonucleic acid
dph	days post-hatch
E1	estrone
E2	17β-estradiol
E3	estriol
EDCs	endocrine disrupting chemicals
EE2	17^{α}-ethinylestradiol
ELISA	enzyme-linked immunosorbent assay
EPA	environmental protection agency
ER	estrogen receptor
GTH 1	gonadotropin hormone-I
GTH 2	gonadotropin hormone-II
GnRH	gonadotropin releasing hormone
H&E	haematoxylin and eosin
Hcl	Hydrochloric acid
LOEC	lowest observed effect concentration
OECD	organisation for economic co-operation and development
PAHs	polycyclic aromatic hydrocarbons
PAS	periodic acid-Schiff
PCBs	polychlorinated biphenyls
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PMSF	phenyl methyl sulfonyl fluoride
RNA	ribonucleic acid
T	testosterone
Tb	17β-trenbolone
TBA	trenbolone acetate
Vtg	vitellogenin
11-KT	11-keto testosterone

ABSTRACT

Endocrine disrupting chemicals (EDCs) are anthropogenic compounds that have similar properties as endogenous hormones in vertebrates and they are capable of interfering with the endocrine system. EDCs have been detected in sewage receiving effluents from industries and livestock feedlots. Fish living in the recipients contaminated with EDCs are reported to have disturbed physiological homeostasis and reproductive disabilities. The deleterious effects have also been reported in amphibians, reptiles, aves and mammals including humans. The natural estrogen, 17β-estradiol (E_2) stimulates the fish liver to synthesize vitellogenin (Vtg), the protein that nourishes the maturation of eggs. 17*α*-ethinyl estradiol (EE_2) is a potent, synthetic estrogen used in oral contraceptive preparations. It mimics E_2 leading to enhanced circulatory Vtg concentrations and male-to-female sex reversal in fish. 17β-trenbolone (Tb), an androgenic steroid, is used as growth promoter in beef cattle. It has been reported to cause declined Vtg concentrations and masculinization in fish. In the present study (Paper I) on juvenile medaka (*Oryzias latipes*), after exposure to 100 ng EE_2/l for 20 days period, there was time-dependent induction in Vtg was noticed. A significant increase in Vtg was observed in 48 hours after exposure. Normal gonadal development in early life stages of male and female medaka was illustrated in time-correlation with Vtg inductions of unexposed fish. The current study (Paper II) has proved the alteration in Vtg levels and sex reversal after experimental exposure to EE_2 and Tb in two model fish species viz., zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*). Complete masculinization of fish after 50 ng Tb/l and complete feminization of fish after 10 ng EE_2/l exposure was observed in zebrafish. In conclusion, zebrafish was determined more sensitive than medaka to both of the test chemicals.

Keywords: 17α-ethinylestradiol (EE_2); 17β-trenbolone (Tb); vitellogenin (Vtg); zebrafish (*Danio rerio*); medaka (*Oryzias latipes*).

BACKGROUND

Endocrine disrupting chemicals /Endocrine disrupters

A number of synthetic industrial chemicals, *i.e*., polycyclic aromatic hydrocarbons (PAHs), organo-chlorine pesticides and pharmaceuticals, *i.e*., 17α-ethinylestradiol have similar properties to endogenous hormones such as estrogens and androgens. Some of these anthropogenic compounds are capable to interfere with the endocrine system of vertebrates and are therefore known as endocrine disruptors (Colborn *et al.*, 1993), commonly referred to as "Endocrine Disrupting Chemicals" (EDCs).

EDCs are ubiquitous in nature. Effluents from industries viz. paper and pulp mills, textile industries, sewage treatment plants etc. are main sources of EDCs. For instance, the estrogens, estrone, 17β-estradiol and 17α-ethinyl estradiol, have been detected in sewage treatment effluents in United Kingdom (Desbrow *et al.*, 1998), Netherlands (Belfroid *et al*., 1999), Germany, Canada (Ternes *et al*., 1999), and Sweden (Larsson *et al*., 1999; Svenson *et al*., 2003). Organo-chlorine pesticides, *i.e*. DDT and lindane have been detected in Indian rivers such as Ganges (Ahmad *et al*., 1996; Kumari *et al*., 2002), Krishna (Sekhar *et al*., 2003) and Kaveri (Rajendran *et al*., 1999). Estrogens and androgens are also found in streams receiving runoff from livestock feedlot effluent (Soto *et al*., 2004), which is another source of EDCs besides industries and sewage treatment plants.

Deleterious effects of EDCs in vertebrates

During the last decade, it has been shown that EDCs cause adverse health effects in different groups of vertebrates, including developmental, neurological, endocrine (Colborn *et al*., 1993) and reproductive alterations (Gray, 1998; Tyler *et al.*, 1998; Janssen *et al*., 1998). Fish living in recipients receiving different types of effluents contaminated with EDCs have reduced reproductive ability (Jobling *et al.*, 1995; Norrgren *et al*., 1998). Global decline in populations has been reported (Houlahan *et al*., 2000) in frogs, for example, *Xenopus laevis* and *Rana pipens* after exposure to a mixture of pesticides (lindane, dieldrin, endosulfan, aldicarb, metribuzine, atrazine) (Christin *et al.*, 2004). The toxic effects on immune system of amphibians have also been reported (Sharma and Reddy, 1987; Luebke *et al*., 1997). A pesticide (DDT complex) spillage into Lake Apopka, Florida, USA caused distorted sex organ development and function in alligators (Guillette *et al.,* 1994). In another group of reptiles, the red-eared slider turtle (*Trachemys scripta elegans*) has also been reported to be causing sex reversal by EDCs (Sheehan *et al.*, 1999). In birds, the reports on severe population declines due to DDE-induced eggshell thinning and DDT complex induced ova-testis have been documented in male Western gulls (Fry, 1995). The mammals, Baltic gray and ringed seals affected with reproductive and immune dysfunctions caused by biomagnification of PCBs in the food chain (Brouwer *et al*., 1989; Vos *et al*., 2000; Van den Belt *et al.*, 2004). Epidemiological studies in humans indicate that there has been decline in semen quality, increased incidence of testicular cancer, cryptorchidism, and

hypospadias over the past 50 years (Carlsen *et al*., 1992). These findings may be related to increased estrogenic exposure in utero (Sharpe and Skakkebaeck, 1993). An increased prevalence of breast cancer have been reported in females exposed to xenoestrogens (Colborn and Clement, 1992; Davis *et al*., 1993; Recchia *et al*., 2004).

Mechanism of endocrine disruption

The environmental compounds affect the biochemical messenger system of fish by acting as agonists or antagonists (Colborn *et al*., 1993). EDCs can alter endocrine function by a variety of mechanisms (Sonnenschein and Soto, 1998) (Fig. 1):

(1) Mimic the effect of endogenous hormones
(2) Antagonize the effect of endogenous hormones
(3) Disrupt the synthesis and metabolism of endogenous hormones
(4) Disrupt the synthesis and metabolism of hormone receptors

Some of the well-known synthetic chemicals and their endocrine disrupting action in fish are reviewed in Table 1:

Table. 1. A list of some familiar synthetic chemicals that have similar properties as endogenous sex hormones and their mechanism of action in fish

Endocrine disrupting action	Chemicals
Estrogenic	17α-ethinylstradiol (EE2), Alkyl phenols (Nonylphenol, octylphenol, Bisphenol A), flavonoids
Anti-estrogenic	Dioxins, Furans, PCBs, Butylated hydroxyanisole (BHA), Pesticides (Aldrin, Allethrin, Dicofol), Dieldrin, Endosulfan, Fenarimol, Fenvalerate, Kepone (Chlordecone), Methoxychlor, Permethrin, Toxaphene, Triadimefon, Triadimenol), Benzophenone
Androgenic	17β-trenbolone, Tributyl tin (TBT)
Anti-androgenic	Flutamide, Pesticides (DDT, Metabolite, Fenitrothion, Iprodione, Linuron, Mirex, Procymidone, Sumithrin, Vinclozolin)
Anti-estrogenic/ anti-androgenic	Lindane, Phthalates [diethylhexylphthalate (DEHP), monoethylhexylphthalate (MEHP), dimethylphthalate (DMP), butylbenzylphthalate (BBP), dibutylphthalate (DBP) and dioctylphthalate (DOP).

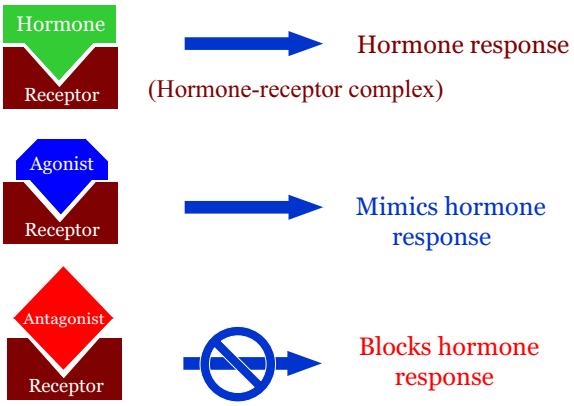

Figure 1. Schematic representation of Hormone-receptor mechanism of endocrine disruption by agonist and antagonist.

Steroids

Steroids are fat-soluble hormones with a tetra cyclic base structure. They enter into cells and bind to receptors to form a steroid-receptor complex. The complex undergoes dimerization, travels to the nucleus of the cell to bind DNA and promotes protein synthesis by gene transcription and translation (Fig. 2). By function, steroids may be divided into the groups, androgens, estrogens, progestogens, anabolics, and catabolics.

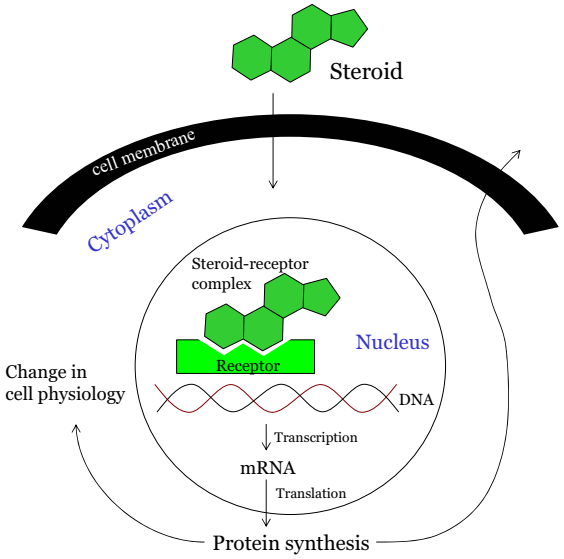

Figure 2. Schematic representation of general mechanism of action of steroids in the cell.

Estrogens

Natural estrogens that are synthesized from the ovary of fish are 17β-estradiol (E_2), estrone (E_1), estriol (E_3) where E_2 is the most potent natural estrogenic compound. A number of phytoestrogens (produced by plants) such as flavonoids (genistein, naringenin, and kaempferol), coumestans (coumestrol), and lignans (enterodiol and enterolactone); mycotoxins (produced by fungi) such as zearalenone are known to affect the endocrine system (Korach *et al*., 1994; Kuiper *et al.,* 1998).

17*α*-ethinyl estradiol (EE_2) is a potent, synthetic, and pharmaceutical estrogen (Folmar *et al*., 2000; Metcalfe *et al*., 2001) commonly used in oral contraceptive preparations. EE_2 excretes in urine in an inactive conjugated form but is readily activated through bacterial activity in the sewage (Guengerich, 1990). Entering the aquatic environment EE_2 is relatively persistent and causes endocrine disruption in fish (Tyler *et al.,* 1998). For example, EE_2 has been measured in sewage treatment plants at concentrations up to 7.0 ng/l in United Kingdom (Desbrow *et al.,* 1998), <0.2–7.5 ng/l in Netherlands (Belfroid *et al*., 1999), 15 ng/l in Germany, 42 ng/l in Canada (Ternes *et al*., 1999), and <0.1-15 ng/l in Sweden (Svenson *et al*., 2003). As an estrogen receptor agonist, EE_2 can interfere with the normal endocrine activities and cause sex reversal in fish (Papoulias *et al*., 1999). The structural similarity of EE_2 with those of natural estrogens is illustrated in Fig. 3.

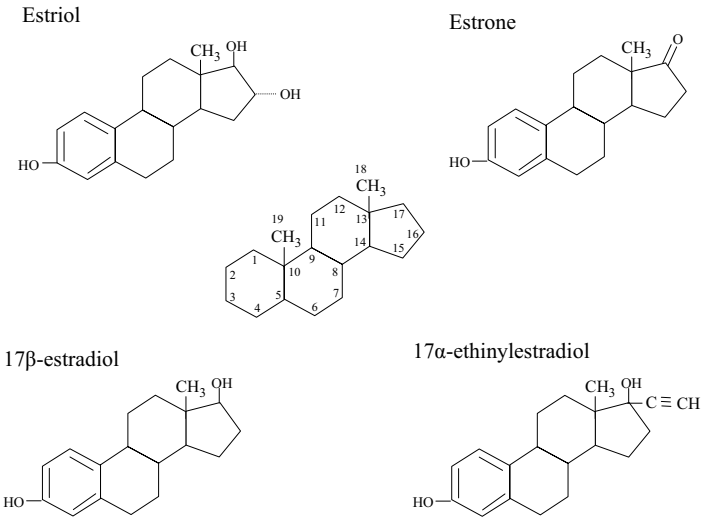

Figure 3. Chemical structures of natural (17β-estradiol, estriol, estrone) and synthetic (17α-ethinylestradiol) estrogens.

Androgens

The male reproductive hormones, androgens, are produced in the testes of fish to contribute secondary sexual characteristics. They include mainly, testosterone (T), androstenedione, 11-ketotestosterone (11-KT), and 11β-hydroxy testosterone. Female fish also produce testosterone, which aromatises to E_2 by cytochrome P450 (CYP19) in the granulosa cells of ovarian follicles (Petersen *et al*., 2000).

17β-trenbolone (17β-hydroxyestra-4, 9, 11-trien-3-one) is an anabolic, androgenic steroid, with the homology to testosterone structure (Fig. 4), can disturb the endocrine homeostasis by mimicking 11-KT and testosterone. Trenbolone acetate (TbA), an acetate form of trenbolone, degrades proteins through reduction in the activity of catabolic glucocorticoids (Schmidely, 1993). It is used as growth promoter in beef cattle in USA and Canada (Lange *et al*., 2001, Wilson *et al*., 2002). The anabolic androgenic steroids in urine are metabolised mostly as glucuronides and sulfates. TbA is excreted mainly as 17α-trenbolone, 17β-trenbolone and triendione in urine (Pottier *et al*., 1981, Wilson *et al*., 2002) (Fig. 5), and enter into environment through the excreta along with downstream from cattle farms. The metabolites of TbA can remain active for more than 270 days in the manure piles (Schiffer *et al*., 2001). TbA has been reported to masculinize channel catfish (*Ictalurus punctatus*) (Galvez *et al*., 1995) and blue tilapia (*Oreochromis aureus*) (Galvez *et al*., 1996). 17β-trenbolone is a potent androgen receptor agonist, caused masculinizing effect, and declined fecundity in fathead minnow (*Pimephales promelas*) (Ankley *et al*., 2003).

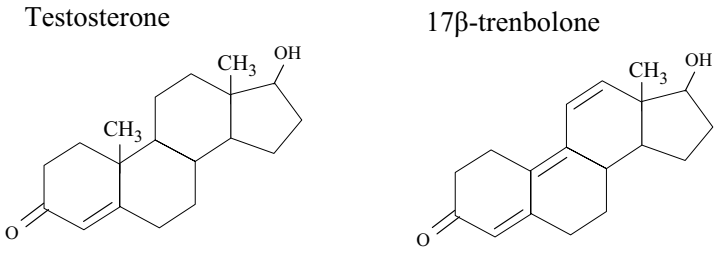

Figure 4. Chemical structure of natural androgen (testosterone) and anabolic steroidal androgen (17β-trenbolone).

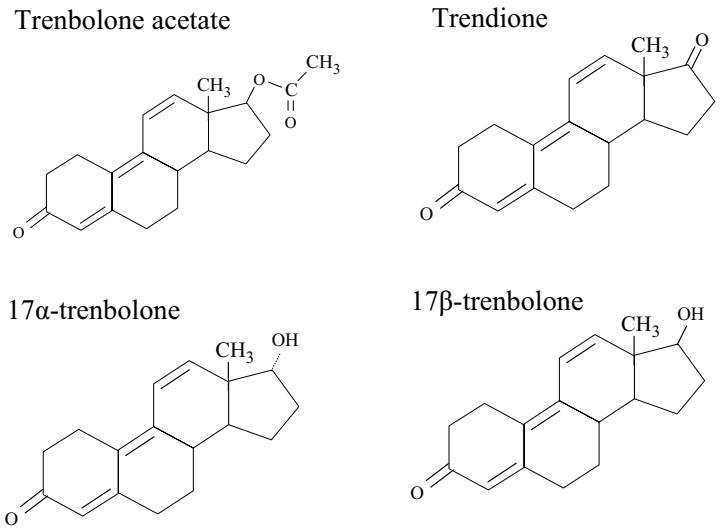

Figure 5. Chemical structures of trenbolone acetate and its metabolites (17α-trenbolone, 17β-trenbolone and triendione).

Impact of EDCs on fish

Some industrial chemicals released into the aquatic environment have been reported to interfere with the hormonal control of sexual differentiation in fish (Kime, 1998&1999) with signs of endocrine disruption (Jobling *et al*., 1998). A number of studies indicate adverse effects of endocrine disruptors in many species of marine, fresh water, and estuarine feral fish. Reproductive abnormalities have been reported in the flounders, *Platichthyes flesus* (UK) (Allen *et al*., 1999) and *Pleuronectes yokohamae* (Japan) (Hashimoto *et al*., 2000). Different species of wild fish affected with intersex condition include roach (*Rutlius rutilus*) in UK (Jobling *et al*., 1998; Minier *et al*., 2000), the shovelnose sturgeon (*Scaphirhynchus platyorynchus*) from the Mississippi River in USA (Harshbarger *et al*., 2000) and white perch (*Morone americana*) from the lower Great Lakes region of Canada (Kavanagh *et al*., 2004). The prevalence of intersex was also exhibited in species, the eelpout (*Zoarces viviparous*), the three spined stickleback (*Gasterosteus aculeatus*) and the perch (*Perca fluviatilis*) from northeastern Germany rivers (Gercken and Sordyl, 2002), the barbel (*Barbus plebejus*) from a tributary in Italy (Vigano *et al*., 2001), and the gudgeon (*Gobio gobio*) (Van Aerle *et al*., 2001) from recipients in UK.

Experimental studies have been performed on different species of fish by the artificial exposure to synthetic chemicals that have similar properties to endogenous sex steroids. Different studies on Japanese medaka (*Oryzias latipes*) revealed disturbed sex differentiation after exposure to 17α-hydroxyprogesterone, androstenedione, androsterone, testosterone propionate (Yamamoto, 1968), methyl

testosterone (Papoulias *et al*., 2000), and EE_2 (Papoulias *et al*., 2000, Scholz & Gutzeit, 2000). In addition, reproductive disabilities such as testis-ova after exposure to p-nonylphenol (Gray & Metcalfe, 1997) and inhibited spermatogenesis after exposure to 4-tert-octylphenol (Gronen *et al*., 1999) in medaka were documented. Exposure of zebrafish (*Danio rerio*) to a mixture of PCBs (Örn *et al*., 1998), ethinylestradiol (EE_2), and 4t-octylphenol (Van den belt *et al*., 2001) caused adverse effect on the reproductive success.

Fish models for evaluation of EDCs

A variety of fish species have been used as test organisms for the detection of EDCs. This means that interpretation of results and extrapolation between species may be difficult due to variation in sensitivity between species. The largest group of vertebrates, the teleost super-order, comprises more than 90% of the total number of described fish species. In order to harmonise test guidelines based on fish it is essential to develop mutual test protocols suitable for a small number of species. Today, OECD (Organisation for Economic Co-operation and Development) promotes three small laboratory freshwater species as model test species. They are zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*), and fathead minnow (*Pimephales promelas*). A short description of the test species is given below:

Zebrafish

Danio rerio (Hamiton-Buchanan, 1822/1823), a tropical Cypriniform (family Cyprinidae) is commonly called zebrafish because of its striped integument. The zebrafish is native of India (Ganges and Brahmaputra rivers) and extended to Pakistan and northern Burma. The zebrafish is a regular spawner normally ovulating approximately every fifth day. They are characterized by high fecundity providing between 100-500 eggs at each occasion. The eggs are non-adherent, transparent and have a developmental period from fertilization to hatching of 96h at 26°C (Laale, 1977). According to Takahashi (1977), male zebrafish pass through a phase of juvenile hermaphroditism. All fish start to develop ovaries at the age of 10–12 days and the period of sex differentiation begins at the age of approximately 23–25 days where in some of the fish the ovaries degenerate and transform into testes. The process of sex differentiation completes approximately at 40 days post-hatch and the final maturation of the gonads will generally be finished at an age of 60 days.

Japanese Medaka

Oryzias latipes, a freshwater killifish (family Cyprinodontidae), is native to the rice paddies of Japan, Taiwan, and Southeast Asia and is commonly called Japanese medaka or Medaka. They produce 10 to 30 eggs per spawning. In medaka, the undifferentiated gonad directly differentiates into either testis or ovary because of its' stable genetic XX/XY sex determination system and a sex

determining gene (DMY) is isolated from Y chromosome (Matsuda *et al*., 2002). Medaka is considered as a powerful model species (Environmental Agency, Government of Japan, 2000) with a short reproduction cycle to enable the study of complete life stages in order to probe the effects of EDCs. EDCs have ability to alter the phenotypic sex of fish when exposed prior to or during sexual differentiation and cause sex reversal irrespective of genotype (Yamamoto, 1953; Hunter and Donaldson, 1983). If the medaka d-rR strain is used, genomic sex can be distinguished from functional sex by the hormone insensitive orange colour trait, which is specified by a Y-chromosomal gene (Yamamoto, 1975).

Fathead minnow

Fathead minnow, *Pimephales promelas* is a member of the family Cyprinidae and is widely distributed in North America. The name 'fathead minnow' is derived from the shape of the head (*Pimephales* –'fat head') and it's colour in breeding males (*promelas* – 'forward' and 'black') (Mettee *et al*., 1996). It produces 50-100 adhesive eggs every 3-5 days (Jensen *et al*., 2001) and the eggs hatch in 5 to 6 days. They have distinct secondary sexual characteristics in both sexes.

Fish life cycle assays

Today, OECD considers four new test guidelines for detection of EDCs. These are briefly described as:

1. Fish Screening Assay

The aim of this test is to detect activity of androgens and estrogens including agonist/antagonist and aromatase inhibitors. The test is a non-spawning assay based on exposure of adult fish during 21 days. Common core endpoints for all three species include vitellogenin and gonadal pathology.

2. 42-days Reproductive Fitness Test

The aim of this test is to detect impact of toxicants, including EDCs, by assessment on reproduction success. The assay is initiated with a 21-day non-exposure that is followed by a 21-day exposure period. The spawning capacity is checked daily, the hatching success and early stage mortality are determined for the whole period. At termination of the assay, the adult fish are sampled for analysis of vitellogenin and gonadal pathology.

3. Fish Sexual Developmental Test

The aim of this test is to detect the activity of EDCs by exposure during sensitive periods of the life cycle. The assay is based on exposure of juvenile fish before sex differentiation. The core endpoints include vitellogenin and sex ratios.

4. Complete Life Cycle Test / fish-full-life-cycle-test (FFLT)

The aim of this test is to detect activity of EDCs in the parental fish and in their progeny. Core endpoints include spawning success, offspring survival, and sex ratios.

Biomarkers

The alteration of the normal physiology in response to EDCs and the potency of the xenoestrogens can be demonstrated by using in vivo markers.

Vitellogenin

In oviparous vertebrates including fish, the eggs contain a large amount of yolk in the form of a precursor protein, vitellogenin (Vtg), which is produced by the liver under the control of the endocrine system. In fish brains, the hypothalamus is stimulated by external factors, such as water temperature and photoperiod, to secrete gonadotropin-releasing hormone (GnRH), which in turn stimulates the pituitary gland to secrete two gonado tropic hormones (GTH 1 and 2). GTH 1 and 2 trigger the gonads to release 17β-estradiol (E_2) which binds to E_2 receptors and activates the synthesis of Vtg protein in the liver. Vtg is circulated to ovaries (Bun and Idler, 1983) and is cleaved into lipovitellin and phosvitin to deposit in the oocyte by means of yolk during oocyte development. Vtg is the protein source that nourish in the maturation of the embryo and for developing larva (Mommsen & Walsh, 1988; Selman *et al*., 1989). The synthesis of Vtg is regulated by endocrine system under the control of feed back mechanisms through hypothalamus-pituitary-gonadal-liver axis, E_2, and testosterone (Fig. 6). In general, Vtg is not found in male fish but it can be induced upon estrogenic exposure (Sumpter and Jobling, 1995; Folmar *et al*., 1996; Jobling *et al*., 1996; and Tyler *et al*., 1996). Hence, Vtg has become the most frequently used biomarker. Governmental organizations such as OECD and the US Environmental Protection Agency (EPA) consider Vtg as a core biomarker for the detection of endocrine disrupting effects in different species of fish. The estrogenic potency of EDCs can be determined by quantitative analysis of Vtg. Since Vtg levels in different tissues, i.e. liver, heart and blood plasma correlates (Nishi *et al*., 2002), there are different options to measure Vtg depending on fish species.

Different assays for the detection of Vtg include radio immunoassays (RIA), enzyme immunoassays (EIA), immunohistochemistry using monoclonal as well as polyclonal antibodies, RNA protection assay, and transcript analysis by Northern blotting or polymerase chain reaction (PCR).

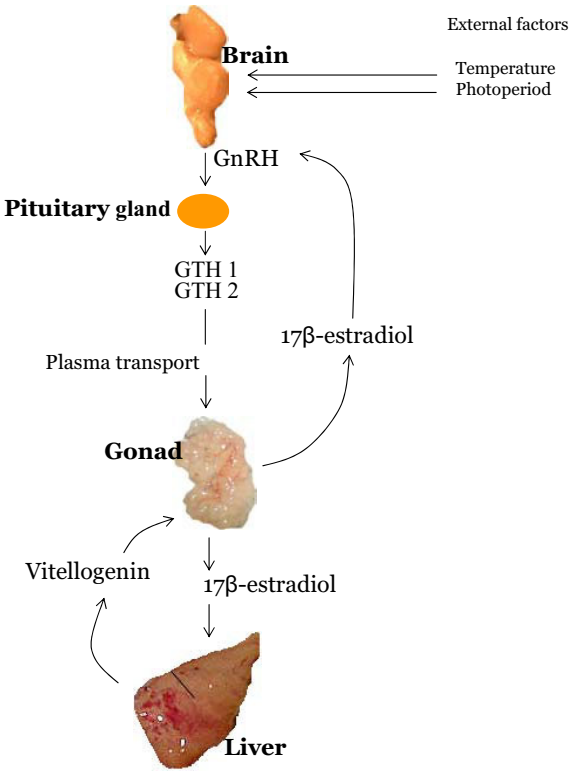

Figure 6. Schematic representation of vitellogenin synthesis in fish and feedback mechanism through hypothalamus-pituitary-gonadal-liver axis.

Fish gonads & histology

Fish have one pair of bilateral gonads: ovaries in females and testes in males suspend from dorsal body wall by means of mesovarium and mesorchium, respectively. Gonads differentiate by the proliferation of primordial germ cells as in other vertebrates. But only a single primordium (cortex) is probably involved in the ontogeny of both ovary and testis (Baroiller *et al*., 1999).

Ovary: A sac shaped organ with ovarian cavity (ovarian lumen) and numerous ovarian lamellae (ovigerous lamellae), is covered by ovarian wall. Teleosts' ovary is generally of cystovarian type (ovarian cavity from each ovary connects to oviduct that lead to the genital pore) as in zebrafish and medaka. Secondly, the semi-cystic type (pocket like structure that opens into funnel shaped transporting groove which leads to the genital pore), which is present in salmonids. Thirdly, the gymnovarian type (ovary spreads like a curtain to release eggs) is present in eel (*Anguilla japonica*).

Cell types in ovary in the order of maturation according to the validation of the fish-screening assay for Endocrine active substances, OECD, 2004:

1. **Oogonium:** It is the smallest cell type with relatively large nucleus, small inapparent nucleolus, and minimal amounts of cytoplasm.
2. **Chromatin nuclear oocyte:** It is surrounded by prefollicular cells. It consists of relatively large nucleus with single large nucleolus and granular cytoplasm.
3. **Perinucleolar oocyte:** It is characterised by multiple nucleoli located at periphery of the nucleus (germinal vesicle).
4. **Cortical alveolar oocyte:** It is larger than perinucleolar oocyte and it can be recognised by the presence of cortical alveoli (yolk vesicles) and distinct chorion (vitelline envelope) between oocyte and perifollicular cells. A mature cortical alveolus consists of distinct central core surrounded by light flocculent material and a membrane. In this stage, irregular enlargement of germinal vesicle and proliferation of pleomorphic nucleoli take place (Selman *et al*., 1993). Yolk vesicles appear earlier than yolk globules and they contain glycoprotein that stain slightly with eosin to give light red colour or deep red with PAS (Takashi Hibiya, 1982).
5. **Early vitellogenic oocyte:** It is larger than cortical alveolar oocyte and characterised by centralised appearance of spherical, eosinophilic, vitellogenic yolk granules or globules that resemble reddish nucleus. Presence of eosinophilic substances i.e. lipoprotein and carbohydrate in yolk globules gives positive staining with H&E but weekly to PAS. Glycerides and cholesterol present in oil droplet that appears as a circular vacuole in paraffin sections but stains black by osmic acid fixation.
6. **Late vitellogenic oocyte:** The increased volume of vitellogenic granules displaces the cortical alveolar material to periphery and nucleus starts migrating to periphery. Germinal vesicle is large and has smooth contour, spherical nucleoli and small nucleoli at centre. Lipid droplets are never seen within zebra fish oocytes (Selman *et al*., 1993).
7. **Mature/Spawning oocyte:** Larger cell type with nucleus migrated to periphery and it appears more hydrated due to vitellogenesis.
8. **Atretic mature oocyte:** This cell type is characterised by zona radiata breakdown and yolk resorption.

There are three layers surrounding the oocyte: inner zona radiata, middle follicle and outer vascularized theca (Willium and Joseph, 1983). Hyperplasia of follicle cells with inner and outer squamous theca cell layers is due to vitellogenesis i.e. accumulation of yolk substances (yolk vesicles, yolk globules, and oil droplets).

Testis: Testes are paired, sac-shaped organs and sperms are carried through sperm duct as in higher vertebrates. In zebrafish, a series of five parallel efferent ducts collects spermatozoa from seminiferous tubules and pass caudo-ventrally to unite at genital papilla (Ewing, 1972; Laale, 1977).

Spermatogenesis: Spermatogonium proliferates to give primary spermatocytes, which undergoes meiotic division leading to secondary spermatocyte, and it further divides to produce spermatids. Finally, spermatids mature to become spermatozoa.

Cell types of testis in the order of maturation according to the validation of the fish-screening assay for Endocrine active substances, OECD, 2004 are:

1. **Spermatogonium:** Largest cell type and consists of vesicular nuclei with distinct nuclear membrane and prominent nucleoli.
2. **Spermatocyte:** Primary spermatocyte is larger than secondary spermatocyte.
3. **Spermatid:** It is smallest cell type with dense nucleus and narrow rims of eosinophilic cytoplasm.
4. **Spermatozoa:** They are mature cells with dark round nuclei and tail.

Along with germinal cells, sertoli cells (cyst cells) which take part in nourishment during the spermatogenesis and interstitial cells (leydig cells) secreting sex steroids are present in testis.

GENERAL AIM OF THE THESIS

The aim of the present study was to determine the impact of endocrine disrupting chemicals on sex differentiation of fish by using two model test species, zebrafish and Japanese medaka, with the exposure of 17α-ethinylestradiol and 17β-trenbolone.

MAJOR CONCLUSIONS

The fish species, zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) are suitable model test species for evaluation of potential endocrine disruption. The two species showed a remarkable sensitivity difference after exposure to 17α-ethinylestradiol (EE_2) and 17β-trenbolone (Tb).

In the time-related study on medaka, a significant Vtg induction was noticed after exposure to 100 ng EE_2/l from 12 dph to 30 dph. The study was elicited that the Vtg induction was increased above the normal Vtg levels in 48 hours after exposure and reached a maximum stable Vtg induction in two weeks. The normal gonadal histology in medaka was illustrated with different stages of gonads and the observed maturation in gonads at 35 dph was correlated with the elevated Vtg levels in control fish between 30 to 38 dph.

The life cycle test with the vitellogenin (Vtg) analysis at 38 dph and sex ratio at 60 dph was resulted in high Vtg induction and complete feminization in zebrafish after 10 ng EE_2/l exposure. Likewise, a significant decline in Vtg was correlated with complete masculinization after 50 ng Tb/l exposure. However, in medaka, the Tb concentration, 50 ng /l was resulted in unaffected sex ratio with no correlation to declined Vtg at 38 dph. Furthermore, regarding EE_2 concentrations, medaka showed the Vtg induction and feminizing effect only at high concentration i.e., 100 ng/l. This delineates a lower sensitivity of medaka when compared to zebrafish towards the estrogen mimic as well as androgen mimic.

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Research paper I

17α-ethinylestradiol (EE_2) induced vitellogenin levels and normal gonad morphology during early life cycle stages in Japanese medaka (*Oryzias latipes*)

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ABSTRACT

Japanese medaka (*Oryzias latipes*) is a cogent fish model to evaluate the potency of xenoestrogens that alter gonad morphology. It necessitates the proper description of normal gonadal development with various stages of cell types. 17α-ethinylestradiol (EE_2), a synthetic potent medicative estrogen, is capable of modifying the sensitive parts of fish life cycle. It greatly influences the body physiology by endocrinal intervention together with reproductive alterations. Vitellogenin (Vtg) is the protein that responds with estrogenic exposure in fish. The study in medaka dealt with Vtg inductions at the regular occasions, 10, 12, 14, 16, 18, 22, 26, and 30 days post-hatch (dph) after EE_2 exposure at 100ng/l for the 20 days period. About 270-fold increase in Vtg production was observed after 48 hrs of exposure. There was a significant ($P < 0.001$) increase in Vtg levels from 12 dph to 30 dph. The study indicates the high sensitivity of medaka to 100ng EE_2/l throughout the exposure period. The developmental stages of gonadal cell types in medaka at 24, 29, 35, 40, 45, 60, 70, and 75 dph were described and they were correlated with the Vtg inductions in control fish.

Keywords: 17α-ethinylestradiol; EE_2; vitellogenin; Japanese medaka; *Oryzias latipes*

INTRODUCTION

Most teleost species are gonochoristic with bipotential gonads, *i.e.,* they cannot be distinguished as either testes or ovaries in advance to the sex differentiation (Nimrod and Benson, 1998; Parker *et al*., 1999). In Japanese medaka (*Oryzias latipes*), the female (XX) and male (XY) sex chromosomal system operates the undifferentiated gonad to differentiate directly into either testis or ovary. However, xenoestrogens may induce sex-reversal in the genotypic males and transform them into functional females (Yamamoto, 1953) when exposed prior to or during sexual differentiation (Hunter and Donaldson, 1983).

17α-ethinylestradiol (EE_2), the hormonal ingredient of many oral contraceptives, is a potent synthetic estrogen (Folmar *et al.*, 2000; Metcalfe *et al*., 2001). Feminization (Papoulias et al., 1999; Scholz and Gutzeit, 2000; Örn *et al*, 2003) and appearance of intersex (ovatestes) gonads (Metcalfe *et al*., 2001; Länge et al., 2001; Seki *et al*., 2002) have been reported as the major consequences in fish after EE_2 exposure. Other effects include suppression of testicular development, testicular fibrosis, and sterility in males (Zillioux *et al*., 2001; and Van Den Belt et al., 2002; Weber *et al*., 2003). In female fish, alterations in ovarian development, ovary size, and reduced egg production are reported as potential effects of EE_2 (Piferrer and Donaldson, 1992; and Zillioux *et al*., 2001; Weber *et al*., 2003; Van Den Belt *et al*., 2003). Vitellogenin (Vtg), an egg protein, is synthesized in liver in response to the hormonal interference of EE_2 when exposed in early/ sensitive stages of life cycle before/ during the sex differentiation. For example, an increase in Vtg induction was indicated in zebrafish (*Danio rerio*) exposed to EE_2 during different developmental periods from hatching to 60 dph (Andersen *et al*., 2003). The plasma Vtg levels rises steadily in female fish during sexual maturation, but the levels rise remarkably in both sexes when exposed to estrogenic chemicals. Therefore, Vtg is one of the most frequently used biomarkers to explore estrogenic activity in oviparous vertebrates (Sumpter and Jobling 1995, Heppell *et al*., 1995, Tyler *et al*., 1996). Sandwich enzyme-linked immunosorbent assay (ELISA) is the favoured enzyme immunoassay (EIA) for precise quantification of whole body Vtg concentrations in fish. Medaka (*Oryzias latipes*) is considered as a powerful model species (Environmental Agency, Government of Japan, 2000) to evaluate estrogenic effects. The present study aimed to determine EE_2 induced Vtg concentrations at regular occasions during early life cycle stages of medaka. Different developmental stages of gonads in unexposed medaka were depicted in order to correlate gonadal maturation with Vtg concentration.

MATERIALS AND METHODS

Test animals

The test animals, medaka fish were obtained from Japan. Adult fish were kept in a tank provided with recycling water system. The eggs of medaka were collected en masse with forceps directly from the vent region where a cluster of eggs remain firm after spawning. The eggs were transferred into glass beakers. After hatching, fifty free-swimming larvae were transferred to experimental 10-liters aquaria.

Test chemicals

The test chemical, 17α-ethinylestradiol (EE_2), was purchased from Sigma Chemical Company®, Sweden and it was dissolved in methanol to make the stock solution at a concentration of 10 mg/l. A concentration of 100ng EE_2/l was used to expose the juvenile medaka from 10 days post-hatch (dph) to 30 dph.

Test procedure

The fish were exposed to EE_2 through an aqueous route starting 10 days after hatching until 30 dph, under semi-static water conditions. A control aquarium was used to compare the fish with the exposed fish. The water was renewed with 50% of the test volume every second day. Aerated dechlorinated tap water was used for the renewal of test volume. The water was at a temperature of 26±2^{o}C, pH range 7-8 and a 12 h dark / 12 h light regime was maintained throughout the test procedure. Air was bubbled into the water through a syringe needle in each aquarium to maintain adequate dissolved oxygen concentration. Fish were fed ad libitum three times daily, once with live *Artemia nauplii* and twice with commercial food (Sera micron®, Nutrafin®, Tetra Min®).

Sampling for Vtg analysis

Five fish were sampled at each occasion viz. 10, 12, 14, 16, 18, 22, 26, and 30 days post-hatch where the first occasion i.e., 10 dph was sampled after 7 hrs of exposure. The control fish were sampled at 10, 20, 30, 34, 38, 46, 48, 50, and 52 days-post-hatch. They were anaesthetized before frozen in liquid nitrogen. The samples were kept at a temperature -80^{o}C until Vtg analysis.

Homogenization and vitellogenin analysis

The frozen whole fish were weighed and homogenized individually in buffer (12ml of Tris Hcl + 2 mg of aprotinin + 120 µl of PMSF) using a manual homogenizer. The volume of the homogenization buffer was added in 10 times the weight of each fish. The homogenate was centrifuged at 16000×g (4ºC) for one hour and the supernatant below the fat layer was collected to determine whole-body homogenate Vtg concentration of each fish. Measurement of Vtg was performed by using a commercially available, pre-coated vitellogenin ELISA kit from Biosense laboratories® (Norway). The procedure was followed according to

the manufacturer's instructions in the booklet provided with the ELISA-kit. The absorbance of samples in the ELISA-plate-wells was measured using microtiter plate reader and the concentration of Vtg in each sample was calculated.

Sampling for histology

In order to perform the histological evaluation of gonads, unexposed fish were sampled at 24, 29, 35, 40, 45, 60, 70, and 75 days-post-hatch. They were fixed in neutral buffered formalin.

Histological preparation

The tail and head of each fish were cut to obtain the required central block of each fish. The blocks were placed in labelled plastic cassettes and then dehydrated using 70% to absolute ethanol, treated with xylene and finally embedded in paraffin. Each paraffin block contained 6-8 individuals. The paraffin blocks were sectioned sagittally on a microtome to get sections of size 3-4 microns. About six sections per fish were cut such that they could show different stages of gonadal development. The sections were transferred on to glass slides and placed on a heating plate for one hour to allow them settle by drying. Then the sections were deparaffinized with xylene and rehydrated using a graded series of ethanol and finally with tap water to make the sections ready for staining by hematoxylin and eosin. After the staining, sections were dehydrated again in ethanol and xylene and then mounted with cover slips to fix them for light microscopy.

Histopathological analysis

The histological sections from the unexposed fish were examined to determine the different cell types of testis and ovary at the different sampling occasions. The sections were pictured using Nikon® digital camera (DXM 1200) attached to the light microscope, Nikon eclipse® E600.

Statistical analysis

The data obtained from vitellogenin measurements were analysed by non-parametric Mann-Whitney *U* test for significant ($P < 0.05$) differences between the control and treatment groups.

RESULTS

Medaka were exposed to a nominal concentration of 100 ng EE_2/l from 10 dph to 30 dph and whole body Vtg levels were measured at regular time intervals. The mean ((±SD) Vtg levels in control and exposed fish are compared in Fig. 1. A significant ($P < 0.001$) time dependent increase in Vtg levels were observed starting from 12 dph to 30 dph.

Histological sections showing various stages of normal gonadal development in medaka at 24, 29, 35, 40, 45, 60, 70, and 75 dph are illustrated in Fig. 2 and Fig. 3. The ovaries at 24 and 29 dph were abounding of perinucleolar oocytes. In testis, spermatogonia were abundant at 24 and 29 dph. At 35 dph, the yolk vesicles were observed in the oocytes of ovary whereas mature sperms were noticed in testis. They are clearly visible at 40 dph. The early vitellogenic oocytes and the late vitellogenic oocytes were observed at 60 and 70 dph respectively. The complete vitellogenesis in oocytes was noticed at 75 dph. The maturation of testis in medaka comprising of different cell types viz. spermatogonia, primary spermatocytes, secondary spermatocytes and spermatozoa are depicted in Fig. 4.

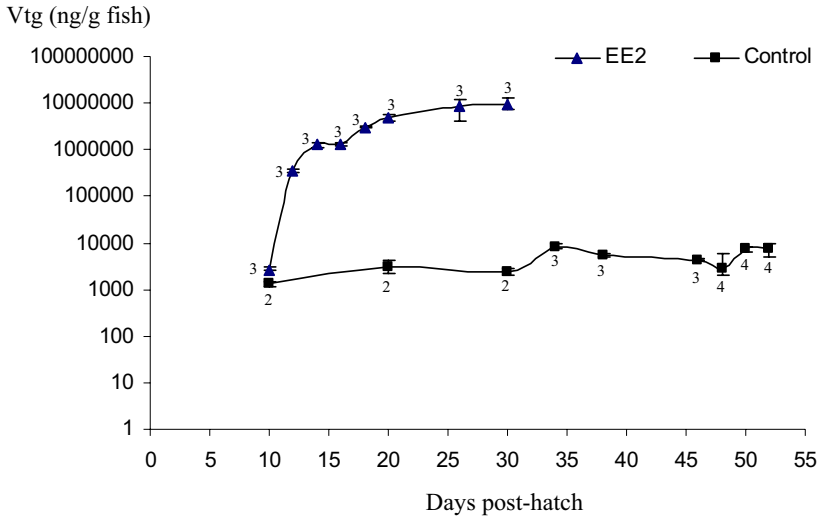

Figure 1. Mean (±SD) vitellogenin (Vtg) concentrations (ng/g fish) at regular time intervals in control [10 days post-hatch (dph) to 52 dph] and 17α.ethinyl estradiol (EE2) exposed (100 ng/l) medaka from 10 dph to 30 dph. The number of fish analysed per occasion is represented along each series.

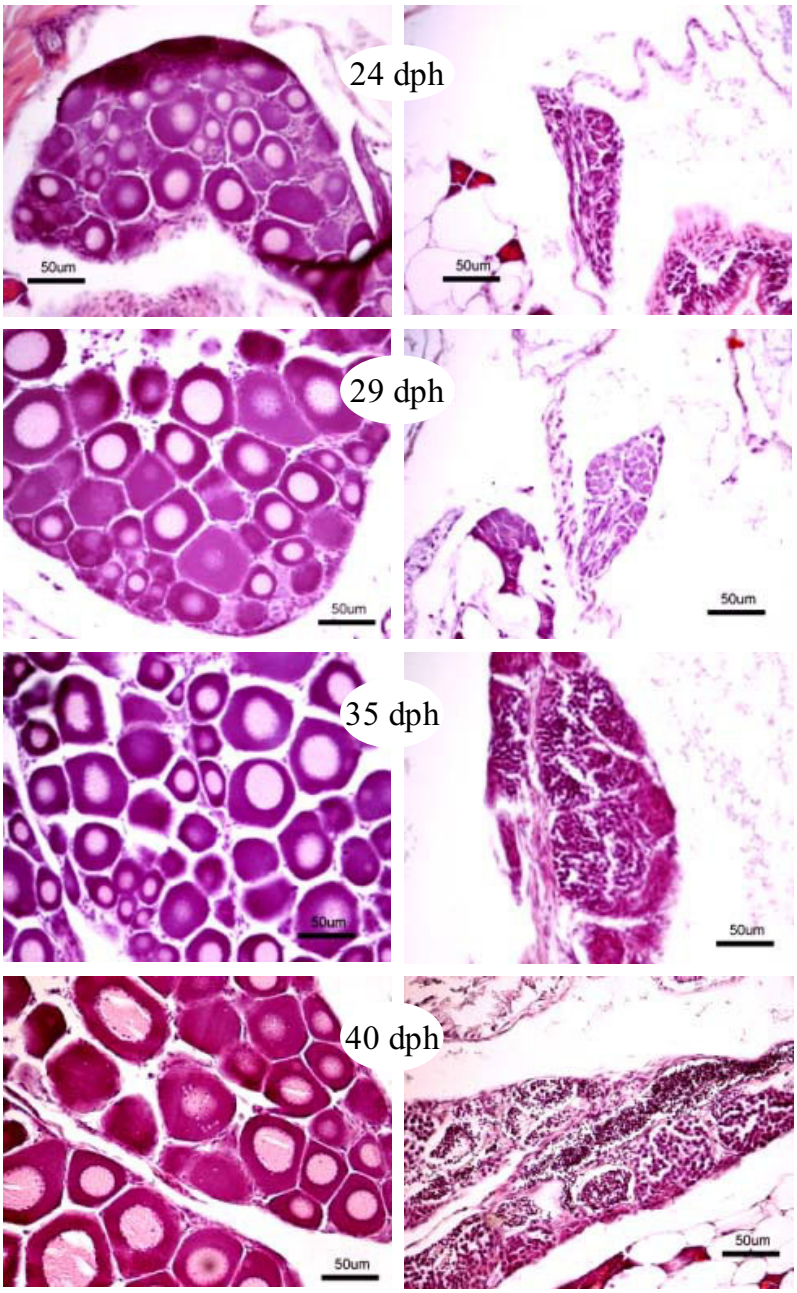

Figure 2. A series of light micrographs showing the normal development of ovary (left side) and testis (right side) at 24, 29, 35, and 40 days post-hatch (dph) in medaka. Bar: 50 µm.

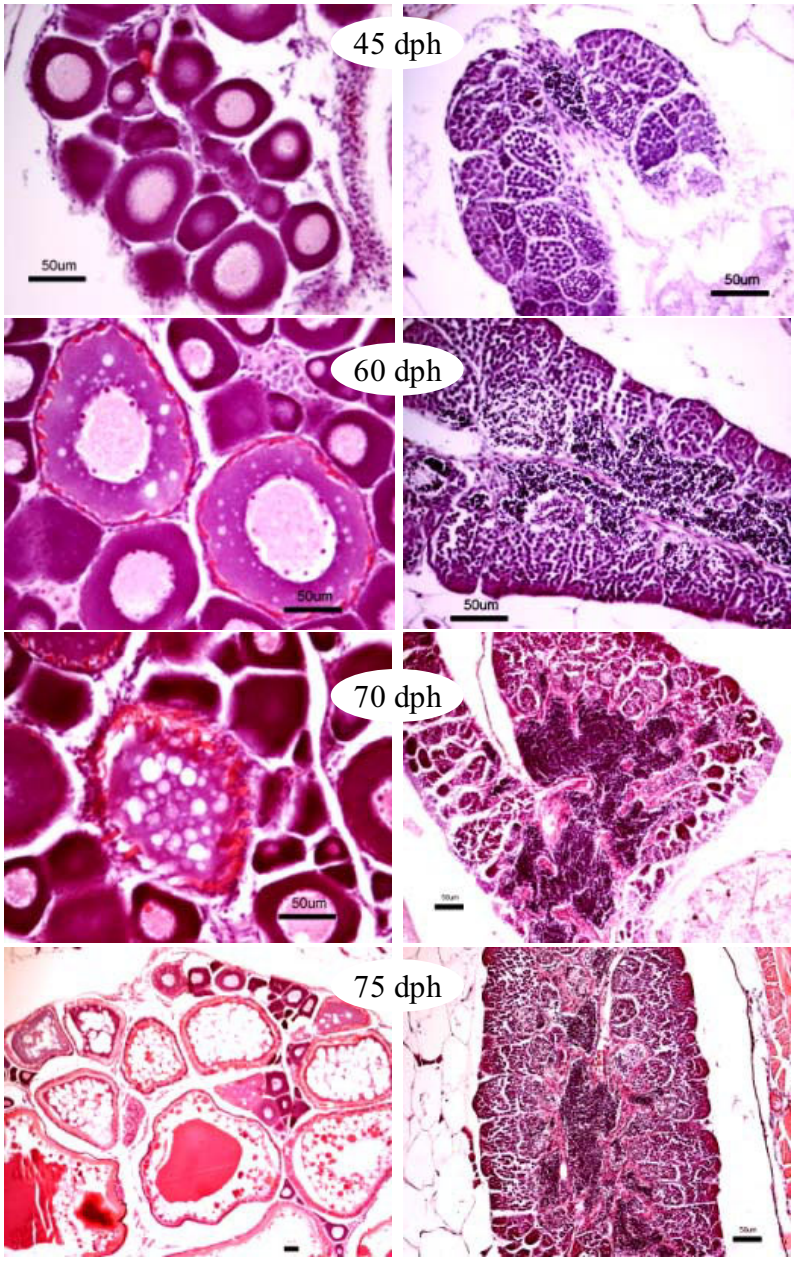

Figure 3. A series of light micrographs showing the normal development of ovary (left side) and testis (right side) at 45, 60, 70, and 75 days post-hatch (dph) in medaka. Bar: 50μm.

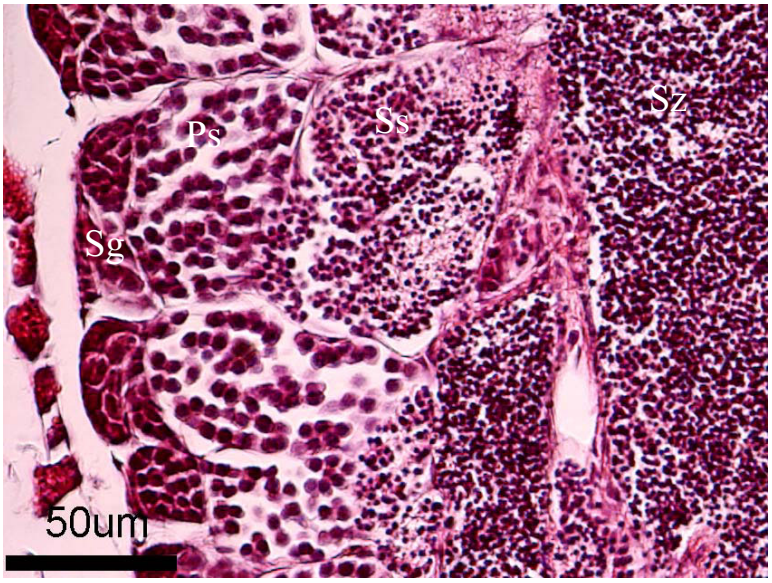

Figure 4. Light micrographs showing the cell types of testis at 75 days post-hatch (dph) in medaka.(Sg- spermatogonia, Ps-primary spermatocytes, Sc-secondary spermatocytes, Sz-spermatozoa. Bar: 50μm.

DISCUSSION

The baseline study on medaka after exposure to 100 ng EE_2/l resulted in a time dependent increase in Vtg levels. The medaka sampled after 7 hours exposure had similar Vtg concentrations to that of the control fish. The Vtg levels were increased continuously from two days of exposure (12 dph) until 20 days (30 dph). Folmar *et al.* (2000) observed Vtg induction approximately 20 mg Vtg/ml plasma in less than 96 hours in sheepshead minnows (*Cyprinodon variegatus*) that exposed to 100 ng EE_2 /l concentration for 16 days. In the present study on medaka, the exposure to 100 ng EE_2/l increased the whole body Vtg levels about 270 times within 48 hours of exposure. Moreover, the observed Vtg concentrations in 2 days exposure period were exceeded the highest observed Vtg concentration of control fish. Thus, the detectable whole body Vtg inductions are apparent between 7 hours and 48 hours after EE_2 (100 ng/l) exposure.

Some of the literatures reported the high Vtg induction in male fish than in females due to EE_2 exposure. For instance, the hepatic Vtg levels were measured higher in male medaka than in female medaka after exposure to EE_2 concentration of ≥ 116 ng/l in adult fish for 21 days (Seki *et al*., 2002). In another study (Schultz *et al.,* 2001), on adult male rainbow trout (*Onchorynchus mykiss*), the plasma Vtg

concentrations were reached the maximum above background levels within 7-9 days after exposure to intra-arterial doses ranging 0.001 to 0.1 mg EE_2/kg. Nevertheless, EE_2 was observed more potent estrogen in fish in responding Vtg with no regard to sex of the individual.

Gonadal development in medaka was observed from 24 dph to 75 dph in regular occasions. The stages, 24 and 29 dph showed the immature gonads with perinucleolar oocytes in ovary and spermatogonia in testis. The appearance of yolk vesicles in oocytes from 35 dph indicates the stage of cortical alveolar oocyte. In testis, the mature sperms were also started to appear at 35 dph. The beginning of gonadal maturation at 35 dph was correlated with the two-fold Vtg production between 30-38 dph of control fish in the time related study. The number of yolk vesicles in oocytes as well as number of mature sperms in testis was increased and clearly visible at 40 dph. The 60 dph stage indicates the early vitellogenic stage with yolk globules. The vitellogenesis of ovary with late vitellogenic oocytes was observed at 75 dph. Unlike the medaka, the late vitellogenic oocytes were detected at 60 dph in zebrafish (paper 2 of this thesis). It is probably due to the early hatching period (2 days) in zebrafish compared to medaka (8-10 days).

In summery, about 270-fold increase in Vtg production was observed after 48 hrs of exposure EE_2 (100 ng/l) and the Vtg concentrations reached to a maximum level after two weeks exposure period. In conclusion, two-days exposure of EE_2 (100 ng/l) is sufficient to cause Vtg induction in medaka whereas increased Vtg levels in control fish between 30-38 dph was correlated with the maturing gonads at 35 dph.

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Research paper II

Comparison of vitellogenin induction and gonad morphology between zebrafish and Japanese medaka after exposure to 17α-ethinylestradiol and 17β-trenbolone

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ABSTRACT

The pharmaceutical estrogen, 17α-ethinylestradiol (EE_2) and the anabolic androgen, 17β-trenbolone (Tb) can interfere with the endocrine and reproductive systems of fish. Potency of these chemicals in zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) was assessed using the core endpoints, vitellogenin (Vtg) and sex ratio at 38 days post-hatch and 60 days post-hatch respectively. Sandwich enzyme-linked immunosordent assay (ELISA) was performed to measure the Vtg levels in fish whole body homogenate samples. High Vtg concentration and feminisation of fish after 10 ng EE_2/l, as well as masculinisation after 50 ng Tb/l exposure were observed in zebrafish. Ova-testis (intersex) condition was observed in medaka exposed to EE_2. A significant (P<0.05) decrease in Vtg production after Tb exposure (50 ng/l) was measured in both zebrafish and medaka. In contrast to the medaka, the high sensitivity of zebrafish to both EE_2 and Tb was clearly indicated in the study.

Keywords: 17α-ethinylestradiol; 17β-trenbolone; vitellogenin; zebra fish; medaka

INTRODUCTION

A number of man-made substances with homology to natural hormones can interfere with the reproductive and endocrine systems causing adverse effects in wildlife and humans (Colborn *et al*., 1993; Gray, 1998; Tyler *et al*., 1998). Reduced reproductive ability was found in fish that are living in recipients receiving different types of effluents contaminated with the substances (Jobling *et al*., 1995; Norrgren *et al*., 1998). Several of studies on different species of fish experimentally exposed to synthetic chemicals with properties similar to endogenous sex steroids has been shown to produce deleterious effects on reproduction. The hormonal active compounds are able to act as agonists or antagonists of the hormonal receptors, thereby causing endocrine disruption (Colborn *et al*. 1993; Sonnenschein and Soto, 1998). One effect of this leads to the disturbance in physiological homeostasis by increased/ decreased circulatory levels of vitellogenin (Vtg), an egg protein synthesized by liver in oviparous vertebrates. For instance, 17α-ethinylestradiol (EE_2), a synthetic medicative estrogen, competes with 17β-estradiol (E_2) to induce Vtg synthesis in fish leading to potentially harmful effects such as intersex (Metcalfe *et al.*, 2001) and feminization (Papoulias *et al*., 1999; Örn *et al*., 2003). 17β-trenbolone (Tb) is an anabolic, androgenic steroid that is used as growth promoter in beef cattle in USA and Canada (Lange *et al*., 2001, Wilson *et al*., 2002). Tb acts as androgen mimic with its high affinity for androgen receptor (Pottier *et al*., 1981; Wilson *et al*., 2002) and eventually lowers the Vtg production. Tb has been repored to masculinize fathead minnow (*Pimephales promelas*) (Ankley *et al*., 2003), channel catfish (*Ictalurus punctatus*) (Galvez *et al*., 1995) and blue tilapia (*Oreochromis aureus*) (Galvez *et al.,* 1996). Biomarkers and bioassays are necessary to detect the endocrine imbalance in fish caused by the anthropogenic chemicals. An in vivo screening test *i.e.,* induction of Vtg (Sumpter and Jobling. 1995; Heppell *et al*., 1995; Tyler *et al*., 1996) in fish has become a valuable tool in laboratory experiments for the investigation of the potency of various chemicals. Some teleost fish species, such as zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*), are considered as model test species for future risk assessment of chemicals.

The present study was aimed to compare the response in zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) by applying core end-points, i.e., vitellogenin and sex ratios, after exposure to model compounds *viz*. 17α-ethinylestradiol (EE_2) and 17β-trenbolone during a sensitive part of the life-cycle.

MATERIALS AND METHODS

Test animals

Adult zebrafish aged 6–8 months were purchased from a commercial dealer. The fish were adapted to laboratory conditions for two months. A group of fish were placed in circular stainless steel spawning funnels provided with a net separating

the fish from the laid eggs. Newly fertilized eggs were collected within two hours after the onset of light. The eggs were transferred into glass beakers until the larvae become free swimming and then they were transferred to 10 litres stock aquaria.

The medaka fish were obtained from Japan and were kept in a tank provided with recycling water system. The eggs of medaka were collected en masse with forceps directly from the vent region where a cluster of eggs remain firm after spawning. The eggs were transferred into glass beakers. After hatching fifty free-swimming larvae were transferred to experimental 10-liters aquaria.

Test chemicals

The test chemicals, 17β-trenbolone (Tb) and 17α-ethinylestradiol (EE_2) were purchased from Sigma Chemical Company®, Sweden and were dissolved in methanol to make the stock solutions at a concentration of 10 mg/l. The exposure of EE_2 was performed in 10 ng/l and 100 ng/l for medaka, whereas only the low concentration i.e. 10 ng/l was used for zebrafish. Trenbolone was used in concentrations of 10 ng/l and 50 ng/l for both species.

Test procedure

The fish were exposed to the chemicals through an aqueous route starting one day after hatching until 60 days post-hatch, under semi-static water conditions. The water was renewed with 50% of the test volume every second day. Aerated dechlorinated tap water was used for the renewal of test volume. The water was at a temperature of 26±2^{o}C, pH range 7-8 and a 12 h dark / 12 h light regime was maintained throughout the test procedure. Air was bubbled into the water through a syringe needle in each aquarium to maintain adequate dissolved oxygen concentration. Fish were fed ad libitum three times daily, once with live *Artemia nauplii* and twice with commercial food (Sera micron®, Nutrafin®, Tetra Min®). All exposures, including controls, were performed in duplicates with 50 fish per aquarium.

Sampling

From each aquarium, five fish were sampled at 38 days post-hatch and were frozen in liquid nitrogen. The samples were kept at -80^{o}C until Vtg analysis. At 60 days post-hatch, all the remaining fish were sampled, fixed in neutral buffered formalin and processed for light microscopy.

Homogenization and vitellogenin analysis

The frozen whole fish was weighed and homogenized individually using homogenization buffer (12ml of Tris Hcl + 2 mg of aprotinin + 120 µl of PMSF)

in 10 times the weight of each fish. The homogenate was centrifuged at 16000×g (4ºC) for one hour and the supernatant below the fat layer was collected individually to determine whole-body homogenate Vtg concentrations. Measurement of Vtg protein in the supernatant samples was performed by using a commercially available, pre-coated vitellogenin ELISA kit from Biosense laboratories® (Norway). The procedure was followed according to the manufacturer's instructions and the absorbance of samples in the ELISA-plate-wells was determined using microtiter plate reader and Vtg concentration whole body homogenate for each fish was calculated.

Histological preparation

The tail and head of each fish were cut to obtain the required central portion and placed in labeled plastic cassettes. They were dehydrated using 70% to absolute ethanol, treated with xylene and finally embedded in paraffin. Each paraffin block contained between 6-8 individuals. The paraffin blocks were sectioned sagittally on a microtome to get sections of size 3-4 microns. About six sections per fish were cut. The sections were transferred on to glass slides and placed on a heating plate for one hour to allow them settle by drying. Then the sections were deparaffinized with xylene and rehydrated using a graded series of ethanol and finally with tap water to make the sections ready for staining by hematoxylin and eosin. After the staining, sections were dehydrated again in ethanol and xylene and then mounted with cover slips to fix them for histological examination using light microscope.

Histopathological analysis

The processed histological sections of fish were examined to determine the sex and gonadal development of each fish. The number of oocytes at different stages was counted and the percentage of each cell type was calculated. In testes, the stages of spermatogenesis were examined. The sections were pictured using Nikon® digital camera (DXM 1200) attached to the light microscope, Nikon eclipse® E600.

Statistical analysis

The data obtained from vitellogenin measurements were analysed by non-parametric Mann-Whitney *U* test for significant ($P < 0.05$) differences between the controls and the the exposed groups. Fisher's Exact Test was used to recognize differences in sex ratios between control and each treatment group.

RESULTS

EE_2 exposure

The measured mean Vtg concentration in zebrafish exposed to 10ng/l of EE_2 was 4986622 ng Vtg/g fish (Fig. 1), whereas in medaka it was 2414 ng Vtg/g fish (Fig. 2). The mean Vtg concentration in medaka exposed to 100 ng EE_2 /l was 1424432 ng Vtg/g fish. In relation to control groups, the Vtg levels were highly significant (***$P < 0.001$) at concentrations of 10 ng EE_2 /l and 100 ng EE_2 /l in zebrafish and medaka respectively. In zebrafish, male (M) to female (F) sex ratio was 0:62 after exposure to 10 ng EE_2 /l (Fig. 4). The corresponding ratio in medaka was 24:22 (M: F) ((Fig. 5). One fish with ovatestis i.e., presence of ovarian cells in atrophied testis was observed. The number of male and female fish after each treatment was listed in table 1. Medaka exposed to 100 ng EE_2 /l resulted in 53 females, one male and six ovatestes fish (Fig. 6). Late vitellogenic oocytes were observed in EE_2 treated groups of medaka. The suppressed gonads after EE_2 exposure were depicted from the haematoxylin & eosin stained sagittal sections of zebrafish (Fig. 7).

17-β-trenbolone exposure

The Vtg concentrations were 478 ng Vtg /g and 386 ng Vtg /g in trenbolone (Tb) exposed zebrafish at concentrations of 10ng/l and 50ng/l respectively (Fig. 1) whereas in medaka they were 1539 ng Vtg /g fish and 813 ng Vtg /g fish (Fig. 2). A significant decrease in Vtg , relative to the control groups (*p <0.05) was observed in both zebrafish and medaka exposed to 50 ng Tb/l. The sex ratios (M: F) in zebrafish were 20: 51 and 65: 0 at 10ng and 50ng trenbolone/l respectively. The corresponding ratios (M: F) in medaka were 54: 51 and 51: 59. The number of male and female fish after each treatment was listed in table 1.

Comparison of Vtg induction between zebrafish and medaka

The quantified Vtg concentrations in control zebrafish and medaka were 546 ng Vtg /g and 2119 ng Vtg /g fish, respectively. Compared to medaka, relative Vtg concentration was very high in zebra fish exposed to 10ng EE_2/l. No significant difference in Vtg levels was observed between zebrafish and medaka after exposure to either concentrations of trenbolone (Fig. 3). Complete sex reversal was resulted in zebrafish towards males at 50ng Tb/l and towards female side at 10ng EE_2/l. In medaka, ova-testis condition was detected in EE_2 exposed individuals. The ovarian cell types, perinucleolar oocytes, cortical alveolar oocytes and early vitellogenic oocytes were counted about 86%, 7% and <1% respectively in zebrafish control groups. The corresponding values in medaka were 93%, 4%, and <1% respectively.

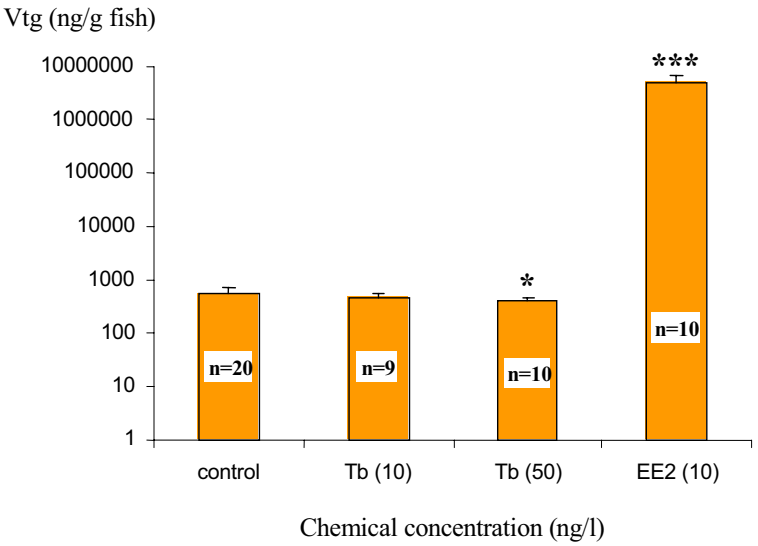

Figure 1. Mean (±SD) vitellogenin (Vtg) concentrations at 38 days-post-hatch in zebrafish exposed to different concentrations of 17β-trenbolone (Tb) and 17α-ethinylestradiol (EE_2). Significant differences are shown with asterisks (*P value < 0.05 is considered as significant). The number of individuals analyzed per group is given inside each bar.

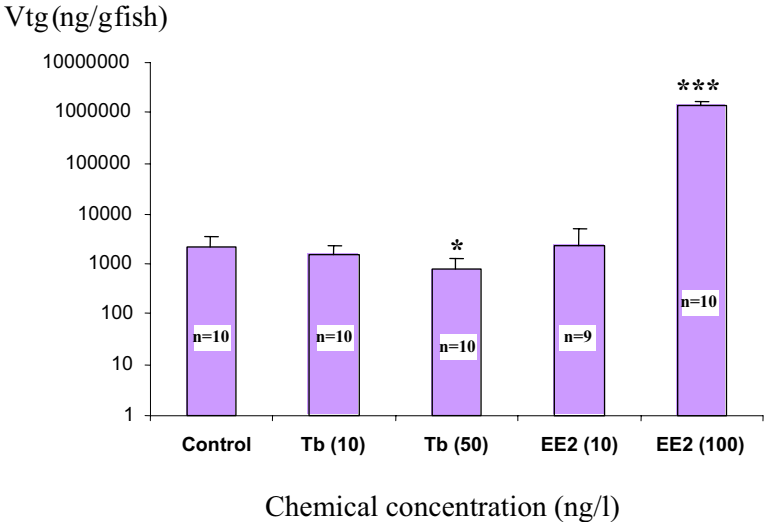

Figure 2. Mean (±SD) vitellogenin (Vtg) concentrations at 38 days-post-hatch in medaka exposed to different concentrations of 17β-trenbolone (Tb) and 17α-ethinylestradiol (EE_2). Significant differences are shown with asterisks (*P value < 0.05 is considered as significant). The number of individuals analyzed per group is given inside each bar.

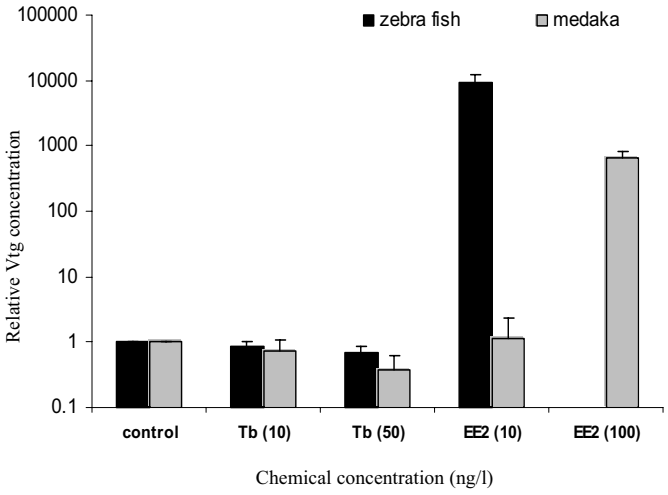

Figure 3. Comparison of relative vitellogenin (Vtg) concentration between zebrafish and medaka after exposure to 17β-trenbolone (Tb) and 17α-ethinylestradiol (EE2)

Table 1. The number of males and females in unexposed and exposed [17β-trenbolone (Tb), 17α-ethinylestradiol (EE_2)] zebrafish and medaka from one day post-hatch (dph) to 60 dph

Zebrafish	Males	Females	Ovatestis	Total
Control	26	52	-	78
Tb (10 ng/l)	20	51	-	71
Tb (50 ng/l)	65	0	-	65
EE_2 (10 ng/l)	0	62	-	62

Medaka	Males	Females	Ovatestis	Total
control 1	55	54	-	109
Tb (10 ng/l)	54	51	-	105
Tb (50 ng/l)	51	59	-	110
Control 2	28	36	-	64
EE_2 (10 ng/l)	24	22	1	47
EE_2 (100 ng/l)	1	53	6	60

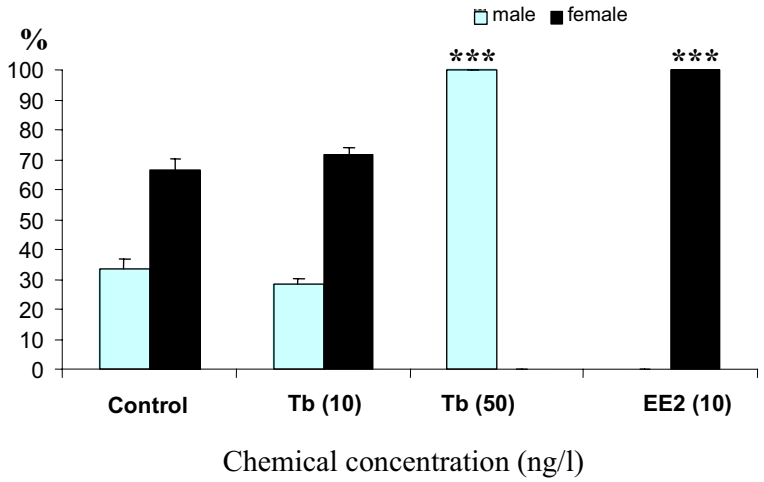

Figure 4. Mean percentages (± SD) of male and female zebrafish after exposure to different concentrations (ng/l) of 17β-trenbolone (Tb) and 17α-ethinylestradiol (EE2) from 1 day post-hatch (dph) until 60 dph. The asterisks (*** $P < 0.001$) represent the significant difference from the control group.

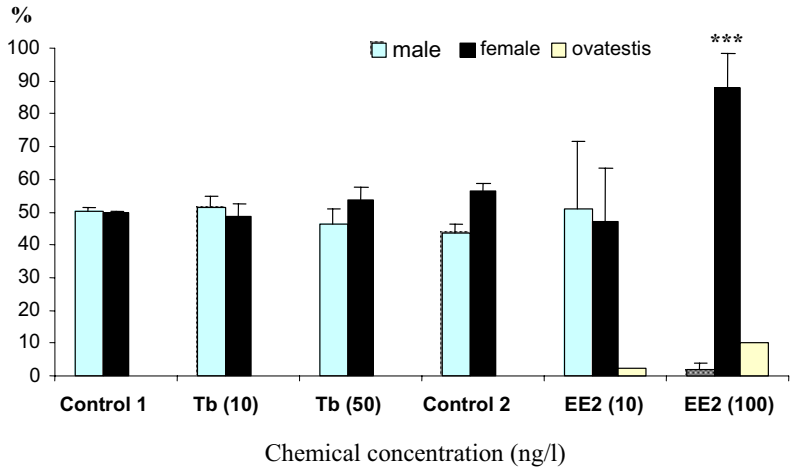

Figure 5. Mean percentages (± SD) of male and female medaka after exposure to different concentrations of 17β-trenbolone (Tb) and 17α-ethinylestradiol (EE2) from 1 day post-hatch (dph) until 60 dph. Control 1 belongs to Tb group and control 2 belongs to EE2 group. The asterisks (*** $P < 0.001$) represent the significant difference from the control group.

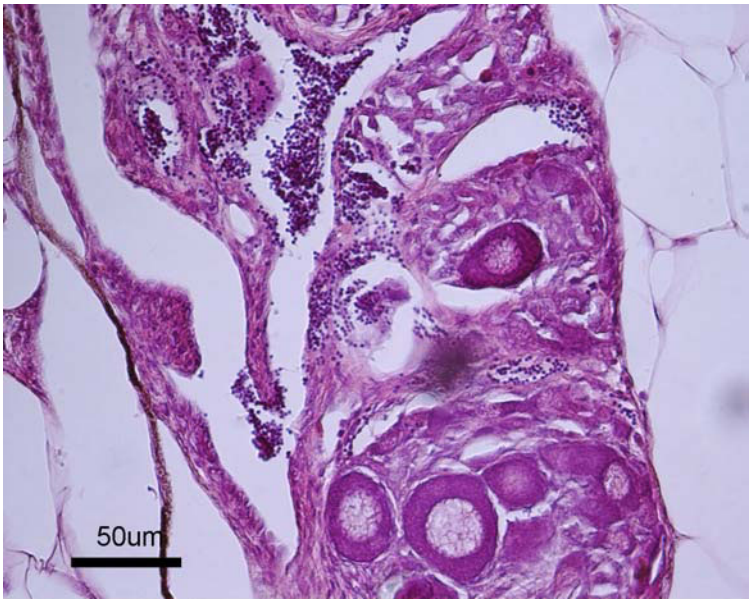

Figure 6. Light micrograph showing the ovatestis in medaka after exposure to EE2 (100 ng/l) for 60 days post-hatch (dph). Bar: 50µm.

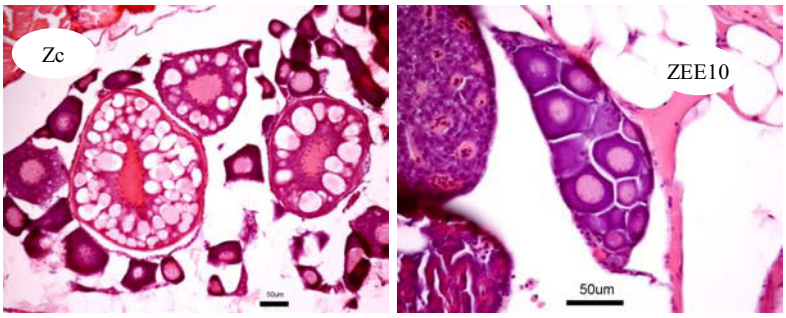

Figure 7. Light micrograph showing the early vitellogenic oocytes in control zebrafish (Zc) in comparison with perinuclear oocytes in EE_2 (10 ng/l) exposed zebrafish (ZEE10). Bar: 50µm.

DISCUSSION

Recent studies investigating fish in response to EDCs during the early developmental period indicate that there are considerable differences in sensitivity among fish species. Genetic variation and disparate patterns of sexual differentiation are probable causes for the interspecific differences in sensitivity. Nevertheless, an alteration in sensitivity also depends on the route of exposure (*i.e*., aqueous, dietary, direct injection through intra-muscular or intra-peritoneal route) and exposure system viz. flow-through, semi-static/ static renewal system.

The present study clearly shows the difference in sensitivity between the zebrafish and the medaka to EE_2 exposure and supports the statement that zebrafish is a highly estrogen sensitive member of the cyprinid family (Rose *et al*., 2002). As an estrogen agonist, EE_2 binds to the estrogen receptor (ER) by imitating 17β-estradiol. This leads to enhanced levels of circulatory vitellogenin (Vtg). The lowest observed effect concentration (LOEC) of EE_2 for Vtg induction in zebrafish was 1.67ng/l after 21 days exposure (Fenske *et al*., 2001) and 2.97ng/l after 8 days exposure (Rose *et al*., 2002) periods. Vtg induction is highly variable and influenced by several factors such as duration of exposure, stage of life cycle and species. Örn *et al.* (2003) indicated the dose dependent Vtg production in EE_2 exposed zebrafish from 20 dph to 38 dph at concentrations 2-25ng/l. A significant (P<0.05) increase in Vtg production was indicated during different exposure periods between hatching to 60 dph of zebrafish exposed to 15.4±1.4ng EE_2/l (Andersen *et al*., 2003). However, diversified results are associated with indifferent experimental conditions of the test protocol with regard to water temperature, pH, photoperiod etc (Baroiller *et al*., 1999).

Van den belt *et al*. (2003) reported that the measured Vtg concentrations in EE_2 exposed (hatch to 3 months) zebrafish whole body homogenates were significant at 10 (**P<0.01) and 25 ng/l (***P<0.001). However, in the present study (hatch to 38 dph), the Vtg induction was highly significant (***P < 0.001) even at the concentration of 10ng EE_2 /l when compared to unexposed group. Örn *et al.* (2003) reported that the mean Vtg concentration was nearly one million ng/g fish in EE_2 (10ng/l) exposed zebrafish form 20 dph to 38 dph, whereas the Vtg concentration in the current study (hatch to 38 dph) was about five million ng/g fish. This clearly indicates the enhanced Vtg induction when EE_2 exposure was initiated right from hatching. In contrast, the adult zebrafish are also pliable to respond to EE_2 exposure irrespective of their sex. For instance, about 200-fold increase in Vtg levels was found when adult male zebrafish were exposed to 10ng EE_2/l for 7 days (Holbech *et al.,* 2001). Petersen *et al.* (2000) reported the induced Vtg concentration in adult zebrafish as approximately 2270000 ng /g fish after exposure for 30 days at 20 ng EE_2/l.

With regard to the medaka, the LOEC of EE_2 for hepatic Vtg induction in exposed adult males was 63.9 ng/l (Seki *et al.,* 2002). In the present medaka study, there were two exposure concentrations *i.e*., 10 and 100ng/l to determine the whole

body Vtg inductions. However, the lower concentration caused no Vtg induction, whereas the higher concentration induced 1000-fold compared to controls.

There are ample instances of reports regarding Vtg induction and sex reversal in other species of fish after artificial exposure to EE_2 at wide range of concentrations. In juvenile fathead minnow (*Pimephales promelas*), Vtg was induced by 2 ng/l EE_2 (Panter *et al*., 2002) and Schultz *et al.* (2001) reported the increased concentrations of Vtg about 17000 times above background levels in adult male rainbow trout (*Onchorynchus mykiss*) using intra-arterial exposure doses from 0.001 to 0.1 mg EE_2/kg.

In order to study potential effects of EE_2 on gonad histology, the exposure was performed until 60 dph. EE_2 exposure has been reported to cause permanent male-to-female sex reversal (Papoulias *et al*., 1999) and modification of gonad morphology. Sex ratio is the crucial endpoint to determine feminization in EE_2 exposed fish. The present study on zebrafish exposed to 10ng EE_2/l from one dph to 60 dph was proved the skewed sex ratio in female direction i.e., 100% females, supporting the previous study (20dph to 60 dph) by Örn *et al*. (2003). However, no ovatestis was detected in the sex-reversed fish. Regarding ovarian development, it has been reported that the presence of oogonia and previtellogenic stages of ovarian follicles and suppressed gametogenesis in 2-60-dph exposed (10ng EE_2/l) zebrafish (Weber *et al*., 2003; Hill and Janz, 2003; Andersen *et al*., 2003). The current study also displayed retarded ovarian development at 60-dph.

In medaka exposed to 10ng EE_2/l, sex ratios were 22/48 (male M) and 26/48 (female F), respectively (Scholz and Gutzeit, 2000). In the present medaka study, the corresponding values were 24/47 (M), 22/47 (F) and one fish with testis-ova condition was observed. Metcalfe *et al.* (2001) observed the testis-ova condition at 0.1 and 1 ng EE_2/l and the sex ratios at 10 ng EE_2/l were 23/49 (M), 25/49 (F) after 100 dph exposure. Therefore, medaka is pliable to intersex condition at low EE_2 concentrations despite the insignificant effect on sex ratio. The high EE_2 concentration, *i.e.,* 100 ng/l resulted in feminization as well as intersex condition in medaka. In the study by Scholz and Gutzeit (2000), 100% sex reversal to females but no intersex gonads were observed at 100 ng EE_2/l in medaka exposed for 2 months. In contrast, with the same concentration of EE_2 and exposure period in medaka, the present study was resulted in 88% females, 10% intersex gonads and one male fish. Similarly, a study by Metcalfe *et al.* (2001) showed 43/49 (91%) females and 4/49 (8%) testis-ova after 100 ng EE_2/l treatment from hatch to 100 days. The considerable appearance of intersex fish clearly shows the male to female sex reversal in early life period after EE_2 exposure at 100 ng/l. The observed late vitellogenic oocytes in EE_2 treated groups indicate the maturation of gonads when compared to control groups. Moreover, EE_2 exposures are reported to cause intersex in mature fish, *i.e*., after complete sex differentiation. For instance, in adult male medaka aged 6 months, the intersex condition was indicated after exposed to $\geq$63.9 ng EE_2/l for 21 days (Seki *et al*., 2002).

Intersex in other species of fish has also been reported after EE_2 exposure. In sheepshead minnow (*Cyprinodon variegatus*), the LOEC for induction of testis-

ova was 20 ng EE2/l (Zillioux *et al*., 2001). A male:female ratio of 5:84 with intersex in 11 % of fish was observed in fathead minnow (*Pimephales promelas*) at 56 days post hatch after exposure to 4 ng EE_2 /l (Länge et al., 2001).

The growth promoter in cattle, trenbolone has recently been reported to produce masculinizing effects in fish. Ankley *et al.* (2003) noticed that a minimum concentration of 27ng Tb/l can cause a significant decrease in Vtg concentrations, masculinizing effect and decline in fecundity in female fathead minnow (*Pimephales promelas*) after exposure for 21 days. In the present study, zebrafish and medaka were exposed to 17β-trenbolone in order to assess the crucial endpoints, Vtg and sex ratio. The higher concentration of trenbolone, *i.e.,* 50 ng/l resulted in significant decrease in Vtg levels in both zebrafish and medaka at 38 days post hatch. Contradictory, the sex ratios varied between the two species. In zebrafish, the higher concentration caused skewed sex reversal in male direction, whereas no effect on sex ratio was observed in medaka. It elucidates the suppressive effect of trenbolone at physiological level despite the unapparent phenotypic expression in medaka. The lower concentration (10 ng/l) of trenbolone caused no significant effect on either Vtg or sex ratios in both the species. The higher concentration (50 ng/l) indicates the possible trenbolone associated androgen mimicking endocrine disruption in zebrafish with regard to Vtg as well as sex ratios.

The present study was purposed to compare the potential effects of Tb and EE_2 with similar concentrations for both the test species. However, a study by Hill and Janz (2003) reported that EE_2 induced high mortality rate (>90%) in zebrafish exposed to 100 ng/l during 2–60 dph. In fact, unlike the medaka, the EE_2 concentration, 100 ng/l is highly toxic in zebrafish and it is impractical to assign the sex ratio with few survivals after exposure. Thus, the high EE_2 concentration was not used in the present zebrafish exposure study to compare with medaka. The background mean Vtg levels in medaka were about 4 times higher to that of zebrafish at 38 days post hatch. It is probably due to the difference in hatching time i.e., 8 days in medaka and 3 days in zebrafish.

In summary, the relative Vtg concentration in zebrafish was observed approximately 10000 times higher than medaka after exposure to 10 ng EE_2 /l. Moreover, there was 100% feminization in zebrafish at the same concentration. Similarly, exposure to 50 ng/l of trenbolone resulted in 100% masculinization. Therefore, the difference in sensitivity between zebrafish and medaka with regard to the core endpoints, Vtg and sex ratio was apparent. In conclusion, the present study disclosed that the zebrafish is highly sensitive to estrogen mimics as well as androgen mimics when compared to the medaka.

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