FACULTY OF FISHERIES AND PROTECTION OF WATERS
UNIVERSITY OF SOUTH BOHEMIA IN ČESKE BUDĚJOVICE

Sperm Functions Impairments and Steroidogenesis Transcriptomic Alternations in Fish Exposed to Endocrine Disrupting Chemicals

Vliv endokrinních disruptorů na funkčnost spermií a změny ve steroidogenezi transkriptomik u ryb

Azadeh Hatef

Czech Republic, Vodňany, 2012

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I, Azadeh Hatef, thereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.

In Vodňany 5th of May, 2012

Supervisor:

Prof. Dipl.-Ing. Otomar Linhart, D.Sc.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Zátiší 728/II
389 25 Vodňany
Czech Republic

Consultant:

M.Sc. Sayyed Mohammad Hadi Alavi, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Research Institute of Fish Culture and Hydrobiology (RIFCH)
Zátiší 728/II
389 25 Vodňany
Czech Republic

Head of Laboratory of Reproductive Physiology, USB, FFPW, RIFCH:

Dipl.-Ing. Martin Pšenička, Ph.D.

Dean of Faculty of Fisheries and Protection of Waters:

Prof. Dipl.-Ing. Otomar Linhart, D.Sc.

Board of doctorate study defence with referees:

Prof. Dipl.-Ing. Petr Ráb, D.Sc. – head of the board
Prof. Zdeňka Svobodová, DVM, D.Sc. – board member
Assoc. Prof. Dipl.-Ing. Martin Flajšhans, Dr.rer.agr. – board member
Assoc. Prof. Dipl.-Ing. Pavel Kozák, Ph.D. – board member
Assoc. Prof. M.Sc. Jana Pěknicová, Ph.D. – board member
Assoc. Prof. M.Sc. Josef Matěna, Ph.D. – board member
Assoc. Prof. Stanislav Navrátil, DVM, Ph.D. – board member

Prof. Patrick Kestemont, Ph.D. – International referee
Prof. Andrzej Ciereszko, Ph.D. – International referee

Date, hour and place of Ph.D. defence:

12th of September 2012 at 9:30 a.m. at USB, FFPW, RIFCH, Vodňany

Name:	Azadeh Hatef
Title of thesis:	Sperm Functions Impairments and Steroidogenesis Transcriptomic Alternations in Fish Exposed to Endocrine Disrupting Chemicals

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CHAPTER 1

GENERAL INTRODUCTION

1. INTRODUCTION

Aquatic ecosystems (including rivers, estuaries and lakes) throughout the world are repositories for enormous amounts of both natural and man-made chemicals (either industrial or agricultural compounds) containing thousands of chemicals (Kime, 1998; Denslow and Sepulveda, 2008; Gregory et al., 2008). Therefore, in the aquatic ecosystem, as the major repository of environmental pollutants, fish can provide an early warning of effects that may later become apparent in other wildlife and humans (Kime, 1998). Several field studies throughout the world have shown that except in cases of localised acute pollution, dead fish are seen much more rarely. Indeed, fish from polluted environments commonly exhibits alternations in growth, development and reproduction indicating that fish health is being compromised by long-term low-level pollution (Kime, 1998; Gregory et al., 2008).

Since 90s, worldwide considerations have been increased to study the effects of pollutants on fish health, however popular concern has particularly focused on the estrogenic hazards of sewage effluents and the alkylphenolic detergents (Gregory et al., 2008; Denslow and Sepulveda, 2008; Leet et al., 2011). Considering a very wide range of other chemicals released into the aquatic ecosystem from industrial and domestic wastes with potential endocrine disrupting activity, our knowledge is not well enough to understand their modes of action. In this context, both field and laboratory studies will provide valuable information to understand mechanism of toxicity and to determine biological indicators for screening the effects of pollutants on fish health.

2. DEFINITION AND NATURE OF ENDOCRINE DISRUPTING CHEMICALS

The US Environmental Protection Agency has defined *Endocrine Disrupting Compounds* (EDCs) as “an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental process” (Kavlock et al., 1996). The EDCs, therefore, are chemicals with the potential to modify natural endocrine function of humans and other animals. They interfere with the functioning of the endocrine system in at least three possible manners; (a) by mimicking the action of a naturally produced hormone via binding to their hormone receptors, (b) by blocking the receptors in target cells for these hormones and therefore preventing the action of natural hormones, or (c) by altering the synthesis and function of hormone receptors and modifying the synthesis, transport, metabolism and excretion of hormones (Hanet et al., 2008). Over 80,000 chemicals have been introduced into the environment within the last five decades (Leino et al., 2005). Classification of EDCs can be done by chemical structure or their source (Kime, 1998). In most review, they have been classified as heavy metals, pesticides and fungicides, and industrial chemicals including pharmaceutics and sewage effluents and paper mill effluents.

3. MECHANISMS OF ACTION EDCs

EDCs may change endocrine system through receptor mediated mechanisms or receptor independent mechanisms (Denslow and Sepulveda, 2008). Receptor mediated mechanisms have received more attention than other mechanisms, with especial emphasis given to estrogen receptor (ER) mediated pathway (Gregory et al., 2008), but less in known about EDCs with androgen receptor (AR) mediating pathway. EDCs can act both as agonist or antagonist in a dose-dependent manner. For instance, bisphenol A act as ER agonist or antagonist at high and low concentrations, respectively (Safe

et al., 2001). Moreover, EDCs effect can be divided into typical dose-response curve or non-typical dose-response curve. This means that EDCs do not always follow a typical dose-dependent response curve and U-shaped curves can be also observed due to feedback inhibition of endocrine function (Denslow and Sepulveda, 2008).

4. THE REPRODUCTIVE SYSTEM IN FISH

4.1. Morphology of testes and functions

In most fish, the male reproductive system consists of the testes, testicular main duct and spermatic duct (Nagahama, 1983). Testes are containing both germ and somatic cells (Leydig and Sertoli cells) (Nagahama, 1983) (Fig. 1). The testicular main duct and spermatic duct are very important in nutrition of spermatozoa, storage of spermatozoa, synthesis of steroids, and formation of seminal fluid (Alavi et al., 2008). Sperm maturation to gain potentiality for motility occurs in the spermatic duct (Morisawa and Morisawa, 1986).

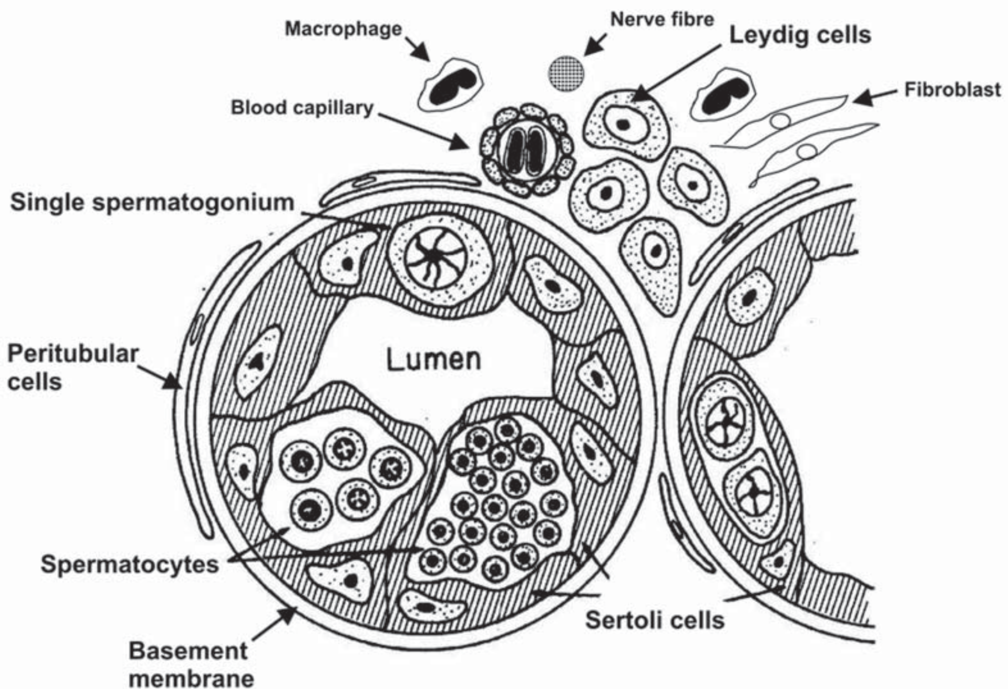

Figure 1. *Scheme of testicular compartments in teleost fish (Vizziano et al., 2008). The figure shows a transversal section of a testis lobule with its lumen. The interstitial compartment is separated from the lobular or germinal compartment by an acellular basement membrane that surrounds the germinal epithelium consisting of Sertoli cells and germ cells. The germinal epithelium is organised in functional units called spermatocysts. Each spermatocyst is formed by Sertoli cells that surround one clone of germ cells at the same stage of differentiation. The interstitium is constituted by steroidogenic Leydig cells, peritubular myoid-like cells that form an incomplete layer over the surface of lobules, blood capillaries, scattered nerve fibres, fibroblasts and macrophages.*

4.2. Endocrine regulation of spermatogenesis and sperm maturation

Reproductive activity in fish is regulated by the endocrine system; hypothalamus-pituitary-gonad axis (HPG). The endocrinological structures and function have been extensively reviewed by Nagahama (1994), Yaron and Sivan (2006), Vizziano et al. (2008), and Watanabe and Onitake (2008). Following environmental stimuli (photoperiod and temperature), hypothalamus releases gonadotropin releasing hormone (GnRH), which stimulates release of gonadotropin (follicle stimulating hormone, FSH and luteinizing hormone, LH) from pituitary. The FSH regulates early testicular development (known as spermatogenesis), while LH controls testicular maturation (known as spermiation).

In most fish, spermatogenesis takes place within testicular cysts formed by Sertoli cells (Nagahama, 1983) and the process of spermatogenesis can be divided into three major phases; the mitotic proliferation of the spermatogonia, the meiosis division of the primary spermatocyte and the transformation of the haploid spermatids into flagellated spermatozoa (Nagahama, 1983). In this context, FSH stimulates the Leydig cells to release 11-ketotestosterone (11-KT), which in turns activates the Sertoli cells to produce activin B. Then, activin B acts on spermatogonia to induce mitosis leading to the formation of spermatocytes and subsequently spermatogenesis (Miura and Miura, 2003). The biological process of spermatogenesis is very complex and there are several other hormones or factors which play critical role; for instance insulin-like growth factors and progestin (17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-P)) (Nader et al., 1999; Miura et al., 2006). At maturity stage, spermatozoa are release from testicular lobules into sperm ducts, where sperm maturation is controled by progestins (17,20β-P or 17α, 20β-21-trihydroxy-4-pregnen-3-one) (Miura and Miura, 2003). At this stage, LH stimulates the production of 17,20β-P in sperm cells, which induces the activation of spermatogenesis related substance 22, homologue of carbonic anhydrase (eSRS22/CA) and finally this enzymatic activity causes an increase in the seminal plasma pH or increase in cAMP of sperm cells (Miura and Miura, 2003).

4.2.1. Steroidogenesis pathway

Testicular steroids play critical roles in regulating testicular development and maturation (Miller, 1988, 2005). Steroidogenic regulation is complex (Fig. 2) and involves positive and negative feedback mechanisms at levels of the HPG (Van der Kraak, 2009). Biosynthesis of sex steroids is similar across vertebrate species and involves a series of steroidogenic enzymes (Payne and Hales, 2004). Steroidogenesis is facilitated by steroidogenic acute regulatory protein (StAR) which plays a rate-limiting role in supplying cholesterol, the precursor for steroids, to the inner mitochondrial membrane (Stocco, 2001). Steroids are synthesized from cholesterol through a series of reactions catalyzed primarily by several cytochrome P450s, including CYP11A (cholesterol side-chain cleavage; P450scc), CYP17 (cytochrome P450 c17a-hydroxylase, 17, 20-lyase), and CYP19A (cytochrome P450 aromatase, A-isoform) as well as by hydroxysteroid dehydrogenases (HSDs), including 3β-HSD, 20β-HSD, and 11β-HSD (Agarwal and Auchus, 2005; Miller, 1988, 2005) (Fig. 2). In human, mice and rat, CYP19 is encoded by the single gene whereas in teleost fish two isoforms of CYP19 are expressed in the brain and gonad. CYP19A is expressed in gonads while CYP19B is expressed in the brain (Callard et al., 2001).

Given the potential for EDCs to influence steroidogenesis through direct or indirect mechanisms (Thibaut and Porte, 2004), and the important role of steroids in regulating the HPG, mRNA transcript of steroid receptors and of genes encoding enzymes in steroidogenesis pathway have been considered important targets to understand EDCs mechanisms of toxicity.

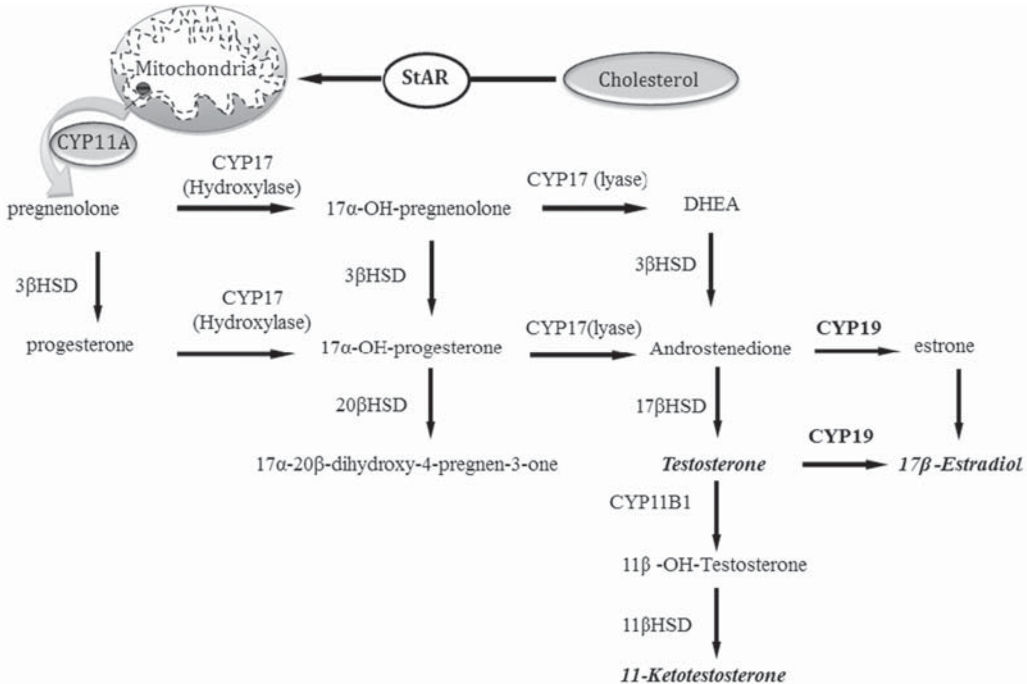

Figure 2. *Steroidogenesis pathway in fish.*

4.3. Sperm biology in fish

Sperm production determined as number of sperm cells released from testicular glands into the sperm ducts as well as formation of seminal plasma during maturity stage (Alavi et al., 2008). These parameters are important in evaluation of fish male fertility, because fertilization success highly depends on sperm/egg ratio (Linhart et al., 2004; Billard et al., 1995).

Fish spermatozoon is differentiated into a head, a midpiece and a flagellum (Lahnsteiner and Patzner, 2008). The head contains the DNA contents for transferring haploid set of the chromosome into the oocyte. Mitochondria are located in midpiece together with the proximal and distal centrioles. Flagellum originates from the basal body and consists of 9+2 pairs of the microtubules. Any damage to sperm morphology may lead to motility dysfunction such as percentage of motility or sperm velocity, which influence male fertility (Linhart et al., 2005; Kaspar et al., 2007).

The spermatozoa of most fish species are immotile in the seminal plasma, due to seminal plasma high concentration of potassium or osmolality (Linhart et al., 1991; Morisawa, 2008). Motility of spermatozoa is induced after release into the aqueous environment during natural reproduction or the diluents during artificial reproduction. It is clear that the motility of sperm is induced due to hypo- and hyper-osmotic pressure in freshwater and marine fishes, respectively (Alavi et al., 2008; Morisawa, 2008).

Duration of sperm motility in fish is very short (from few seconds to less than a few minutes) and its parameters such as beat frequency, ATP content, spermatozoa velocity and the percentage of motile spermatozoa decrease rapidly after triggering the initiation of sperm motility (Alavi and Cosson, 2006; Alavi et al., 2008). Because male fertility in fish highly depends on sperm motility parameters (Billard et al., 1995; Linhart et al., 2005, 2008; Kaspar et al., 2007), studying these parameters could provide valuable information to evaluate toxicity of EDCs on fish reproduction.

5. SELECTED ENDOCRINE DISRUPTORS EXAMINED IN THE PRESENT STUDY

In this section, physico-chemical characteristics of each EDC examined in the present study is reviewed and their environmental level is summarized. In addition, a summary of previous studies on mechanims of action for each EDC is provided.

5.1. Bisphenol A

Physico-chemical characteristics. Bisphenol A (Fig. 3, Table 1), 2, 2-bis (4-hydroxyphenyl) propane, (BPA) is a chemical used primarily in the manufacture of polycarbonate plastic, epoxy resins and as a non-polymer additive to other plastics. BPA contains two phenol functional groups, and it is prepared by the combination of two equivalents of phenol with one equivalent of acetone (Dodds and Lawson, 1936). BPA, with its two benzene rings and two (4, 4')-OH substituents, fits in the ER binding pocket. Biochemical assays have examined the kinetics of BPA binding to ER and have determined that BPA binds both ERα and ERβ, with approximately 10-fold higher affinity to ERβ (Kuiper et al., 1998). However, the affinity of BPA for the ERs is approximately 10,000-fold weaker than that of estradiol (Kuiper et al., 1998; Wetherill et al., 2007).

Environmental concentration. Worldwide production of BPA is about 3 billion kg each year and over 100 tons released into the atmosphere by yearly production (Vandenberg et al., 2009). BPA can be found in wastewater from factories that produce it because it is not completely removed during wastewater treatment. This wastewater containing BPA can be a source of contamination of the aquatic environment (Staples et al., 1998; Kang et al., 2006). BPA levels were reported 0.02–21 and 0.01–8 μg/L in river water (Crain et al., 2007; Kang et al., 2006, 2007). BPA in river waters can be degraded under aerobic conditions but not under anaerobic conditions (see review by Kang et al., 2006). Kang and Kondo (2002) and Kang et al. (2004) found that two bacterial strains (a *Pseudomonas sp.* and a *Pseudomonas putida* strain) with high BPA biodegradability (about 90%).

Mechanisms of action. Several studies have demonstrated that human and wildlife populations exposed to BPA exhibit dysfunctions in reproduction and development (Crain et al., 2007; Wetherill et al., 2007; Vandenberg et al., 2009). Preliminary observations have been suggested that BPA acts as an estrogen receptor agonist, but recent advances show BPA mode of action mediated by androgen receptor antagonistic activity at environmental relevant concentrations (Crain et al., 2007; Vandenberg et al., 2009). This hypothesis opened new insights into anti-androgenic activity of BPA rather than estrogenic mode of action which cause male infertility. However, there are major uncertainties surrounding the spectrum of BPA's mechanisms of action, for instance the tissue-specific impacts. Compared to mammals, less studies has been performed to investigate BPA effects on fish reproduction (Table 2). In fish, most of studies shows estrogenic effects of BPA such as stimulation of vitellogenin (Vtg), however the effective dose are higher than environmentally relevant concentration (Kang et al., 2007). There is one study that shows adverse effects of BPA on sperm quality in brown trout (Lahnsteiner et al., 2005). Moreover, no study has shown inhibition of testicular androgenesis at environmentally relevant concentration. Therefore, some of experiments in the present work were conducted to investigate adverse effects of BPA on fish reproductive physiology at environmentally relevant concentrations.

Table 1. *Physico-chemical characteristics of the selected endocrine disrupting chemicals.*

	Bisphenol A	Di-(2-ethylhexyl) phthalate	Vinclozolin	Mercury chloride
Molecular formula	$C_{15}H_{16}O_2$	$C_{24}H_{38}O_4$	$C_{12}H_9Cl_2NO_3$	$HgCl_2$
Molecular weight	228.29	390.56	286.12	271.52
Water Solubility (mg/L)	120–300	0.041	1,000	36,000 (0 °C) 74,000 (20 °C)
log k_{ow}	3.40	7.50	3.0	
Melting point (°C)	150–155	-50	106–108	276
Boiling point (°C)	220	385		304
LD_{50} mg/kg mammals	3,300–4,240 2,500–5,200	26,000–4,000	> 10,000	> 1
LD_{50} mg/L fish	4.6	> 11.1 µg/L		> 0.03

Morrissey et al., 1987; Staples et al., 1997, 1998; Vandenberg et al., 2009; Weis, 2009

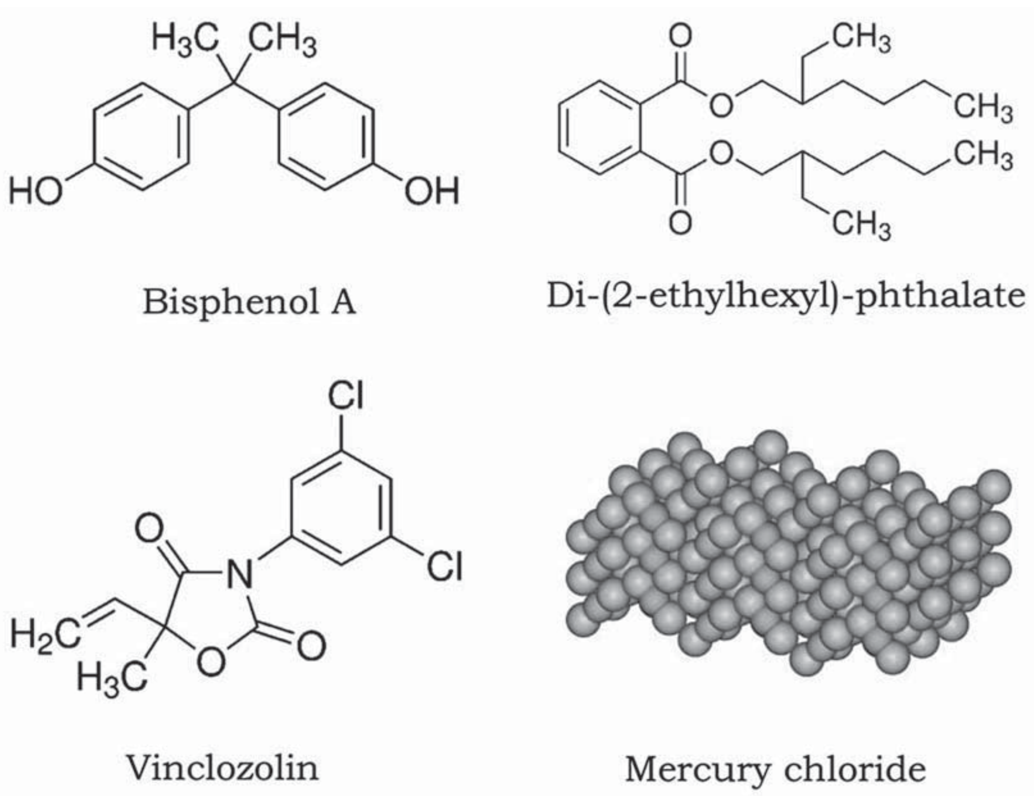

Figure 3. *Chemical structure of selected endocrine disrupting chemicals.*

Table 2. *Bisphenol A and male reproductive physiology in fish.*

Species	Dose (µg/L) (exposure period)	Endpoint	Authors
Guppy	274 and 549 (21 days)	Reduction of total sperm counts	Haubruge et al., 2000
	5; 50; 500; 5,000 (30 days)	Mortality (77%) (5,000 µg/L) Increase in spermatozeugmata, but no spermatogenetic cysts were observed	Kinnberg and Toft, 2003
Fathead minnow	1–1,280 (164 days)	Decrease in GSI (640 and 1,280 µg/L) Increase in plasma Vtg at 169 µg/L following 71 days and at 640 and 1,280 µg/L following 43 days	Sohoni et al., 2001
	119–205 (14 days)	Increase in plasma Vtg	Brian et al., 2005
Medaka	837; 1,720; 3,120 (21 days)	No change in HSI and GSI No change in male fertility Increase in plasma Vtg (3120 µg/L)	Kang et al., 2002
	10 (100 days)	Intersex	Metcalfe et al., 2001
	1,000 (28 days)	Increase in plasma Vtg	Tabata et al., 2004
Zebrafish	1,000 (21 days)	Increase in plasma Vtg	Van den Belt et al., 2003
Brown trout	1.75, 2.40, 5.0 (21 days)	Decrease in sperm density, motility and velocity No change in sperm volume and fertility	Lahnsteiner et al., 2005
Cod	59 (21 days)	Increase in plasma Vtg	Larsen et al., 2006
Turbot	59 (21 days)	Decrease in T and 11-KT Increase in E_2	Labadie and Budzinski, 2006
Carp	1; 10; 100; 1,000 (15 days)	No change in GSI Intersex (100 and 1,000 µg/L) Decrease in T (1,000 µg/L) Decrease in E_2 (1 and 10 µg/L) No change in 11-KT Increase in plasma Vtg (1,000 µg/L)	Mandich et al., 2007
Brown trout	50 (0–63 dpf)	No change in GSI No change in T and E_2	Bjerregaard et al., 2008

Dpf, day post fertilization.

Gonadosomatic index, GSI; hepatosomatic index, HSI; vitellogenein, Vtg; Testosterone, T; 11-ketotestosterone, 11-KT and 17β estradiol, E_2

5.2. *Di-(2-ethylhexyl)-phthalate*

Physico-chemical characteristics. Di-(2-ethylhexyl)-phthalate (DEHP) (Fig. 3, Table 1) is a commonly used plasticizer in polyvinylchloride (PVC) formulations plastics. At least 95% of DEHP produced is applied to this use. There are many other minor applications, including use as a solvent or inert carrier in inks, pesticides and cosmetics, as a vacuum pump oil, for testing air filtration systems and respirators and as the dielectric fluid in electrical capacitors.

Environmental concentration. It has an annual production of approximately 118 million kg, which represent a quarter of all phthalates produced and widespread in the aquatic environment (Petrovic et al., 2001; Planello et al., 2011). The environmental concentration is normally between 2 and 3 μg/L and has been also measured up to 98–219 μg/L in "hotspots" (Fromme et al., 2002). DEHP similar to other phthalate esters is not chemically bound to PVC and can migrate from PVC-containing products to the environment, resulting in significant environmental contamination and human exposure (Heudorf et al., 2007). Under aerobic conditions DEHP is rapidly biodegradable. Its half-life in river water was found to be about one month (Wams, 1987).

Mechanisms of action. It was shown that DEHP is capable of disturbing the reproductive process by mimicking or antagonizing steroid hormone action and its effects on testosterone, luteinizing hormone or estrogen-like activity have been reported in mammal (Akingbemi et al., 2004; Latini et al., 2004). Compared to mammals, there is a significant lack of research investigating the effects of phthalates on the reproductive health of male broodfish (Table 3). No information is available on sperm dysfunctions, but there are a few studies that show transcriptomic alternations of steroid receptors and steroidogenesis mediating genes at environmentally relevant concentrations (Staples et al., 1997; Thibaut and Porte, 2004; Carnevali et al., 2010; Uren-Webster et al., 2010; Crago and Klaper, 2012). This information is essential to establish the mechanisms of toxicity of phthalates in lower vertebrates and results will provide a more comprehensive understanding of the potential threat phthalates pose to the reproductive health of fish in the environment. In one experiment, the effects of DEHP on male reproductive physiology was studied in fish exposed to DEHP at environmentally relevant concentration.

Table 3. *Di-(2-ethylhexyl)-phthalate and male reproductive physiology in fish.*

Species	Dose (μg/L) (exposure period)	Endpoint	Authors
Fathead minnow	12 (28 days)	No change in GSI No change in T Decrease in E_2	Crago and Klaper, 2012
Zebrafish	0.5; 50; 5,000 × 10^3* (10 days)	No change in HSI Decrease in male fertility Lower proportion of spermatozoa in the testes at 50 and 5,000 × 10^3 μg/L	Uren-Webster et al., 2010

Gonadosomatic index, GSI; Hepatosomatic index, HSI; vitellogenein, Vtg; Testosterone, T; 17β-estradiol, E_2
**In zebrafish, DEHP was injected per kg body mass.*

5.3. *Vinclozolin*

Physico-chemical characteristics. Vinclozolin (VZ) (Fig. 3, Table 1) has been registered in the United States since 1981 for use as a fungicide. It is a dicarboximide fungicide widely used for control of diseases caused by *Botrytis cineera*, *Sclerotinia sclerotiorum* and *Monilinia* species in grapes, fruits, vegetables, ornamental plants, and turfgrass (Pothuluri et al., 2000). In mammals, VZ is degraded to two metabolites, M1 (3-[[[3,5-dichlorophenyl]-carbamoyl]oxy]-2-methyl-3-butenoic acid) and M2 (3959-dichloro-2-hydroxy-2-methylbut-3-enanilide) (US EPA, 2000).

Environmental concentration. Presently, use of VZ is decreasing perhaps because of its adverse effects. For instance a total of approximately 61,000 Kg were used in the United States in 1992, which dropped to 55,000 and 25,000 in 1997 and 2002, respectively (http://en.wikipedia.org/wiki/Vinclozolin). Vinclozolin does not undergo significant degradation in sterile water of pH 3, but undergoes fast

degradation at pH 6 and 9. Half-lives at 25 °C and pH 9, 6, and 3 are reported 12 minutes, 61 hours and 70 days, respectively. Two metabolites of VZ (M1 and M2) have half-lives of more than 180 days and are likely highly mobile in the water phase (US EPA, 2000). Bioacumulation rate is very low in fish organs and tissue.

Mechanisms of action. Studies on mammals indicated VZ anti-androgenic activity, whose effects may persist over the next generations. These effects include disruption in testicular development and steroidogenesis as well as decrease in sperm quality (Anway et al., 2005; Elzeinova et al., 2008; Eustache et al., 2009). The VZ effects are mediated by alternations in neuroendocrine regulation of reproduction via inhibiting the binding of the androgens to the AR (Kelce et al., 1997). In fish, studies on VZ effects have been focused on gonadal differentiation and development (Koger et al., 1999; Makynen et al., 2000; Kiparissis et al., 2003) as well as performance of sexual behavior, sexual coloration patterns, sperm counts (Bayley et al., 2002, 2003) (for details see chapter 3.2.). However, no study shows inhibition in testicular androgenesis. There is also a lack of information on VZ potential effects on sperm quality as a critical endpoint for male fertility in fish. Moreover, results in literature on anti-androgenic effects of VZ in fish is somewhat contradictory. Therefore, in the present study, one experiment was conducted to investigate potential anti-androgenic effects of VZ on male reproductive physiology with regard to disruption in testicular steroidogenesis and sperm quality.

5.5. *Mercury chloride ($HgCl_2$)*

Physico-chemical characteristics. Mercury chloride ($HgCl_2$) (Fig. 3, Table 1) is a widespread contaminant in the aquatic environment and is released from various sources, such as the pulp and paper mills, burning of fossil fuels, and agricultural sewage (Crump and Trudeau, 2009; Weis, 2009). It is one of the most toxic forms of mercury because it easily forms organomercury complexes with proteins (Crump and Trudeau, 2009).

Environmental concentration. $HgCl_2$ concentrations in fresh water range from 0.04 to 74 ng/L in lakes and from 1 to 7 ng/L in rivers and streams (Van Look and Kime, 2003). Health Canada has established a human consumption guideline level of 0.5 µg/g wet weight of total mercury in commercial marine and freshwater fish (Crump and Trudeau, 2009).

Mechanisms of action. In mammals, decreases in sperm counts, motility, morphology, and biochemical parameters of the gonads have been reported after exposure to mercury compounds as well as disruption in testicular spermatogenic and steroidogenic functions (Rao and Sharma, 2001; Weis, 2009). In fish, studies have shown that $HgCl_2$ postpones spermatogenesis via inhibitory actions of pituitary gonadotrophs, decreases gonadosomatic index (GSI), and/or inhibits the transformation of spermatids to spermatozoa (Crump and Trudeau, 2009). Effects of $HgCl_2$ on sperm motility and fertilizing ability have been also investigated in a few studies (Lahnsteiner et al., 2004; Van Look and Kime, 2003; Abascal et al., 2007; Dietrich et al., 2010). Despite these findings, the mechanisms of action of $HgCl_2$ on fish spermatozoa still remains unknown.

6. OBJECTIVES OF THE PRESENT STUDY

In the present work, alternations in reproductive physiology and sperm functions were studied in fish or sperm exposed to the selected EDCs, *in vitro* or *in vivo*. The experiments were performed (a) to study potential impacts of selected EDCs on fish reproductive performances, particularly sperm quality as endpoint for male fertility (b) to propose mode of actions by which EDCs affect reproduction

in male fish, (c) to determine whether there are similarities in alternations of mRNA transcript of sex steroid receptors or sex steroid mediating genes among examined EDCs, and (d) to identify potential bimarkers for EDCs examined in the present study.

Chapter 2 contains two studies for understanding toxicity of selected EDCs on sperm functions, *in vitro*. Results suggested that examined EDCs ($HgCl_2$ and BPA) decrease sperm motility or velocity at high concentrations compared to those of exist in the environment. Specific work using $HgCl_2$ showed its toxicity via disruption in energetics suggesting mitochondria as a target and via damage to sperm cells. In these studies, sperm of perch, *Perca fluviatilis* (Percidae; Teleostei) was used.

In Chapter 3 and Chapter 4, male goldfish were exposed, *in vivo*, to selected EDCs (BPA, DEHP and VZ). Potential adverse effects were studied on sperm quality as endpoint for male fertility. Results showed that all of these compounds decrease sperm quality at concentrations approaching environmental levels or slightly higher. For understanding of the modes of action (estrogenic and anti-estrogenic or androgenic and anti-androgenic), sex steroid levels were measured in the blood plasma.

Then after, the mechanisms of action of BPA and DEHP were investigated by evaluating transcripts of sex steroid receptors, and of genes encoding enzymes in steroidogenesis pathway well as vitellogenin mRNA.

Taken together, this work shows reproductive dysfunctions in goldfish exposed to EDCs , *in vivo*. In addition, *in vitro* studies clarify mechanisms of toxicity on sperm cells.

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CHAPTER 2

ENDOCRINE DISRUPTING CHEMICALS AND SPERM FUNCTIONS, *IN VITRO*

2.1. Hatef, A., Alavi, S.M.H., Butts, I.A.E., Policar, T., Linhart, O., 2011. Mechanism of action of mercury on sperm morphology, Adenosine triphosphate content, and motility in *Perca fluviatilis* (Percidae; Teleostei). Environmental Toxicology and Chemistry 30, 905–914.

2.2. Hatef, A., Alavi, S.M.H., Linhartova, Z., Rodina, M., Policar, T., Linhart, O., 2010. *In vitro* effects of Bisphenol A on sperm motility characteristics in *Perca fluviatilis* L. (Percidae; Teleostei). Journal of Applied Ichthyology 26, 696–701.

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MECHANISM OF ACTION OF MERCURY ON SPERM MORPHOLOGY, ADENOSINE TRIPHOSPHATE CONTENT, AND MOTILITY IN *PERCA FLUVIATILIS* (PERCIDAE; TELEOSTEI)

AZADEH HATEF,*† SAYYED MOHAMMAD HADI ALAVI,† IAN A. E. BUTTS,‡§ TOMAS POLICAR,† and OTOMAR LINHART†
†University of South Bohemia, Vodnany, Czech Republic
‡Fisheries and Oceans Canada, St. Andrews, New Brunswick, Canada
§University of New Brunswick, Saint John, New Brunswick, Canada

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Abstract—The main objectives of the present study were to investigate the performance of mercury chloride ($HgCl_2$) on sperm function and structure, identify sites of action of $HgCl_2$, and investigate the mechanism of action of $HgCl_2$ on fish (*Perca fluviatilis* L.) spermatozoa. Direct exposure of nonincubated sperm decreased sperm motility and velocity in a dose-dependent manner and was totally suppressed at 250 μM $HgCl_2$. Adenosine-5′-triphosphate (ATP) content of sperm after activation in an activation medium (AM) containing more than 25 μM $HgCl_2$ did not differ compared with nonactivated sperm. Motility and velocity of demembranated sperm decreased after activation in an AM containing 62 μM $HgCl_2$, and was totally suppressed at 250 μM $HgCl_2$. Incubation of sperm in an immobilizing medium (IM) containing $HgCl_2$ enhanced $HgCl_2$ effects after sperm activation in an AM containing $HgCl_2$. Sperm motility of incubated sperm in an IM without $HgCl_2$ was totally suppressed at 125 μM $HgCl_2$ after 3 h incubation. In case of incubated sperm in an IM containing $HgCl_2$, sperm motility was totally suppressed at 31 μM $HgCl_2$. Adenosine-5′-triphosphate content of sperm was significantly lower in an IM containing $HgCl_2$ greater than 3 μM compared with those of the control (no $HgCl_2$) and lower $HgCl_2$ concentrations. Damage to the plasma membrane and axoneme were observed in sperm incubated in an IM containing $HgCl_2$ compared with the control, when $HgCl_2$ concentration and incubation time increased. In conclusion, $HgCl_2$ acts on sperm through disruption of function of the plasma membrane, axoneme, and ATP content. Environ. Toxicol. Chem. 2011;30:905–914. © 2011 SETAC

Keywords—Adenosine-5′-triphosphate Axoneme Mitochondria Plasma membrane Sperm

INTRODUCTION

Mercury is a widespread contaminant in the aquatic environment and is released from various sources, such as pulp and paper mills, burning of fossil fuels, and agricultural sewage [1,2]. Mercury concentrations in freshwater range from 0.04 to 74 ng/L (0.002×10^{-4}–2.7×10^{-4} μM) in lakes and from 1 to 7 ng/L (0.037×10^{-4}–0.26×10^{-4} μM) in rivers and streams [3]. In mammals, decreases in sperm counts, motility, morphology, and biochemical parameters of the gonads (i.e., adenosine triphosphatase [ATPase] activity) have been reported after exposure to Hg compounds [4]. Mercury compounds have also been shown to affect testicular spermatogenic and steroidogenic functions [2,5,6]. In fish, research has shown that Hg postpones spermatogenesis through inhibitory actions of pituitary gonadotrophs, decreases gonadosomatic index, or inhibits the transformation of spermatids to spermatozoa [2,7–9].

Effects of mercury chloride ($HgCl_2$) on sperm motility and fertilizing ability have been investigated in a few studies [2,10,11]. For example, $HgCl_2$ has been shown to decrease motility of sperm in African catfish, *Clarias gariepinus* [12], brown trout, *Salmo trutta fario* [13], goldfish, *Carassius auratus* [14], and rainbow trout, *Oncorhynchus mykiss* [15], at concentrations of 0.0037, 1.84, 3.68, and 36.8 μM, respectively. Abascal et al. [16] observed no effects of $HgCl_2$ on sperm motility in sea bass, *Dicentrarchus labrax*, when it was added to the activation medium (AM) at concentrations up to 368 μM. Conversely, stronger effects of $HgCl_2$ were observed when sperm were incubated in $HgCl_2$ [14,16]. Despite these findings, the mechanism of action of $HgCl_2$ on fish spermatozoa remains unknown.

In the current study, perch (*Perca fluviatilis* L., Percidae, Teleostei) was used as the model species. Perch is a freshwater carnivorous species, widely distributed in Northern Eurasia and Asia [17]. In their natural environment, male perch mature at two years of age [17]. Sperm morphology and motility dynamics of perch have been studied [18,19]. The spermatozoon differentiates into a head, a midpiece, and a flagellum. Sperm heads lack an acrosome and have an asymmetrical shape as the flagellum inserts mediolaterally on the nucleus [18]. Perch sperm is stored immotile in the seminal plasma at an osmotic pressure of 300 mOsm/kg [18,19]. Mature sperm cells are only activated when they are released into an external hypo-osmotic environment. Sperm motility lasts for a short time, less than 2 min [19]. During this time frame, the spermatozoa must locate an egg and subsequently penetrate the micropyle to achieve fertilization [12,20]. Therefore, the reproductive potential of fish stocks is highly dependent on sperm performance in an aquatic environment [1].

Using three independent experiments, our main objectives were to investigate the effect of $HgCl_2$ on sperm function and structure, to identify sites of action of $HgCl_2$, and to investigate the mechanism of action of $HgCl_2$ on fish spermatozoa. In the first experiment, sperm performance (motility, velocity, and ATP content) was measured in sperm that had been exposed to different concentrations of $HgCl_2$ in an AM. The second experiment studied the effects of $HgCl_2$ on sperm incubated in an immobilizing medium (IM) with or without $HgCl_2$. During 24 h incubation, those that sperm was activated in AM con-

* To whom correspondence may be addressed (hatefa00@vurh.jcu.cz).
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taining $HgCl_2$, and sperm performance was quantified. In the third experiment, the role of the plasma membrane was investigated using demembranated sperm after activation in the presence of various $HgCl_2$ concentrations.

MATERIALS AND METHODS

Sperm sampling

Sperm was collected from mature perch (body length 205–230 mm and weight 110–162 g) in April 2009. Before stripping, the males were anesthetized in clove oil (33 mg/L). Semen was collected in 5-ml syringes by applying slight pressure on the abdomen, from the anterior portion of the testis toward the genital papilla. All attempts were made to avoid sperm contamination by urine, mucus, and blood cells. Semen samples were held at 2°C until processed (within 1 h). Spermatozoa concentration, spermatocrit, and osmolality of the freshly collected samples were measured according to Hatef et al. [21] and were: $61.5 \pm 2.7 \times 10^9$ spz/ml, $67.8 \pm 1.9\%$, and 305.8 ± 1.7 mOsm/kg (mean ± standard error of the mean), respectively.

Sperm activation and assessment of motility

A two-step dilution was used to evaluate sperm motility according to Alavi et al. [19]. First, sperm was diluted in an IM containing 180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, adjusted to a pH of 8.5 (340 mOsm/kg) at a ratio 1:50 (sperm:IM). Next, 1 µl of this diluted sperm was added into 49 µl of an AM containing 50 mM NaCl, 20 mM Tris, adjusted to a pH of 8.5 (110 mOsm/kg). To test the effects of $HgCl_2$, different concentrations of $HgCl_2$ (0.3, 3, 31, 62, 125, and 250 µM) were added to the AM or IM. In all treatments, bovine serum albumin (BSA) was added to the AM before observations at 0.1% (w/v) to prevent sticking of sperm to the glass slide [19]. No BSA was added to the IM, because of its interaction with $HgCl_2$ [16]. Preliminary observations on sperm motility showed no difference between the AM containing $HgCl_2$ and BSA and the AM containing $HgCl_2$ without BSA at different concentrations of $HgCl_2$ (0.3, 3, 31, 62, 125, and 250 µM). In both conditions, with or without BSA, flagellar beating was observed, but forward motion of spermatozoa was observed only using the AM containing BSA. Therefore, in the AM without BSA, forward motion of spermatozoa was inhibited because of stickiness of sperm on the glass slide. After adding $HgCl_2$, pH of the activation and immobilizing mediums ranged from 8.03 to 8.13 for the highest (250 µM) and lowest (0.3 µM) concentrations, respectively. The pH values between 7.0 and 8.5 have been shown to have no significant effect on sperm motility (S.M.H. Alavi, unpublished data).

Sperm motility was recorded by using a 3 CCD video camera (SONY DXC-970MD) mounted on a darkfield microscope (Olympus BX50) equipped with a 20× objective lens and stroboscopic light at a frequency 50 Hz [19,22]. All video recordings were done at room temperature. After recording, the analyses of sperm motility and velocity were based on the successive positions of sperm heads from video frames, using a videorecorder (Sony DVO-1000 MD). Using a micro-image analyzer (Olympus Micro Image 4.0.1. for Windows, CASA), five video frames were captured in real time from the DVD recorder and accumulated (overlapped) using CASA. The final output of the micro-image analyzer shows positions of motile spermatozoa heads in five different locations and marks them as red-green-green-green-blue, whereas the immotile spermatozoa appear in white. The percentage of motile spermatozoa was calculated by counting the red or blue head locations versus the number in white [22]. To measure sperm velocity, the distances of five head positions (distance between red and blue) were measured and divided by the time spent moving such a distance [22]. For each treatment, two to three independent activation trails were conducted. The mean of the independent activation trials was used for statistical analysis.

Experiment 1—Exposure of nonincubated sperm to $HgCl_2$

To evaluate the instantaneous effect of $HgCl_2$ on motility and velocity, we exposed sperm to the AM containing various concentrations of $HgCl_2$. Sperm from 12 males was prediluted in the IM that contained no traces of $HgCl_2$. Immediately after dilution, sperm was activated in the AM. Diluting sperm is an important technique for evaluation of sperm motility, because highly dense sperm in the AM may lead to an incorrect evaluation [22]. Next, 1 µl of this sperm suspension was pipetted in the AM containing 0.3, 3, 31, 62, 125, and 250 µM $HgCl_2$ (0.085, 0.85, 8.5, 17, 34, 68 mg/L, respectively). The control was AM without $HgCl_2$. We later quantified ATP content of sperm in low (0 and 31 µM) and high (125 and 250 µM) doses of $HgCl_2$ in the AM (methodology outlined in later sections).

Experiment 2: Exposure of incubated sperm to $HgCl_2$

Sperm from four males was incubated in the BSA-free IM for up to 24 h at 2°C. At times 0, 3, 6, and 24 h postincubation, sperm motility and velocity were evaluated in two different ways: incubated sperm in the $HgCl_2$-free IM (0.0 µM $HgCl_2$) was activated in the AM containing $HgCl_2$ (0.0, 0.3, 3, 31, 62, 125, and 250 µM), and incubated sperm in the IM containing different $HgCl_2$ concentrations (0.0, 0.3, 3, 31, 62, 125, and 250 µM) were activated in an AM containing the same concentrations of $HgCl_2$ (0.0, 0.3, 3, 31, 62, 125, and 250 µM $HgCl_2$). The ATP contents of incubated sperm either without (0 µM) or with (0.3–250 µM) $HgCl_2$ were determined. Samples were collected for studying morphological changes of sperm using electron microscopy (methodology outlined in later sections).

Experiment 3: Demembranation and reactivation of sperm

To determine whether $HgCl_2$ influences axonemal structure, demembranated sperm was used, and its motility was activated in the AM containing $HgCl_2$ at concentrations of 62 and 125 µM. One microliter sperm was diluted in 49 µl of a demembranation solution composed of 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid, 0.5 mM ethylene glycol tetraacetic acid, 20 mM Tris-HCl, and Triton X-100 0.04% (w/v) adjusted to a pH of 8.2. Then, 1 µl of this suspension was added to an AM containing 100 mM NaCl, 1 mM dithiothreitol, 0.5 mM ethylene glycol tetraacetic acid, 20 mM Tris-HCl, 1 mM Mg-ATP, and $HgCl_2$ (62 and 125 µM) [16,23]. The control was AM without $HgCl_2$.

Electron microscopy

In experiment 2, sperm was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer and kept at 4°C. The samples were postfixed and washed repeatedly for 2 h in 4% osmium tetroxide at 4°C and dehydrated through an acetone series. Samples for scanning electron microscope were dehydrated at the critical point dryer Pelco CPD 2 (Ted Pella). Sperm samples were coated with gold under vacuum with an SEM Coating Unit E5100 (Polaron Equipment) and observed using a JSM 6300 (JEOL, Akishima). Samples for transmission electron micro-

Table 1. Summary of statistics ($F_{NDF,DDF}$) obtained from one-way analysis of variance (ANOVA) used to compare effects of $HgCl_2$ on sperm motility and velocity at each time postactivation[a]

	Time postactivation (s)				
Parameter	15	30	45	60	90
Sperm motility	$172.5_{5,40.1}$	$120.8_{5,39.0}$	$75.2_{5,36.8}$	$59.7_{5,38.4}$	$84.0_{5,35.0}$
Sperm velocity	$21.8_{5,37.1}$	$27.1_{5,38.6}$	$32.1_{5,37.3}$	$41.5_{5,29.8}$	$29.3_{5,20.8}$

[a] In this experiment, sperm was directly exposed to different concentrations of $HgCl_2$ in an activation medium.
All values $p < 0.0001$.

scopy were embedded in resin (Polybed 812). A series of ultrathin sections were cut using a Leica UCT ultramicrotome (Leica Mikrosysteme) and double-stained with uranyl acetate and lead citrate. Samples were viewed in a JEOL 1010 (JEOL) operated at 80 kV.

ATP content

The ATP content was determined by using the bioluminescence method described by Perchec et al. [24]. In the first experiment, sperm of four individuals was used to measure ATP contents during the period of activation. Samples of sperm that had been activated in AM containing 0, 31, 125, and 250 μM $HgCl_2$ were collected for evaluating ATP at 0, 30, 45, and 60 s after sperm activation. After sperm activation, 500 μl of this suspension was diluted in 5,000 μl boiling medium at each sampling time and left for 2 min at 98 to 100°C. The boiling medium contained 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, 10 mM magnesium acetate, 2 mM ethylenediaminetetraacetic acid, and 3 mM sodium azide, pH 7.75. In the second experiment, ATP content was measured in incubated sperm at different times postincubation with or without $HgCl_2$. Incubated sperm in IM (50 μl) was added to 2.5 ml boiling medium and left for 2 min at 98 to 100°C. Next, the sperm suspension was centrifuged for 15 min at 14,000 rpm. The supernatant was removed and stored at −80°C until further analysis. The procedure for ATP determination of sperm involved the addition of purified luciferin-luciferase Bioluminescence Assay Kit CLS II (Roche Diagnostics). Luminescence was read with a multifunctional micro plate reader infinite M200 (TECAN). The ATP content of each sperm sample was calculated using the standard method provided in the kit and was reported as nanomoles ATP per 10^9 spermatozoa.

Statistical analyses

All data were analyzed using SAS statistical analysis software (ver 9.1; SAS Institute). Residuals were tested for normality (Shapiro-Wilk test; Proc Univariate) and homogeneity of variance (plot of residuals vs predicted values; Proc Gplot). As needed, data were transformed to meet assumptions of normality and homoscedasticity. Velocity and ATP content were $\log_{10}$-transformed, whereas percentage data (motility) was arcsin square-root transformed. Alpha was set at 0.05 for main effects and interactions. Each male was considered a replicate. All data are presented as mean ± standard error of mean.

Experiment 1: Exposure of nonincubated sperm to $HgCl_2$

To evaluate the instantaneous effect of $HgCl_2$, we exposed sperm to an AM containing various concentrations of $HgCl_2$. Sperm motility and velocity in the different AM concentrations were analyzed using a single-factor analysis of variance (ANOVA) (Proc Mixed; SAS Institute) [25]:

$$Y_{in} = \mu + M_i + \varepsilon_{n(i)} \text{ Model 1}$$

where μ is the true mean; M_i is the effect of $HgCl_2$ concentration (where $i = 0$–125 μM); and $\varepsilon_{n(i)}$ is the residual error. Separate ANOVAs were run at each postactivation time.

Experiment 2: Exposure of incubated sperm to $HgCl_2$

To evaluate the effects of $HgCl_2$ on incubated sperm in BSA-free IM without or with $HgCl_2$, sperm motility was activated in an AM containing various concentrations of $HgCl_2$. Sperm motility, velocity, and ATP content were analyzed using repeated-measures mixed-model ANOVAs (PROC MIXED; SAS Institute, 2003):

$$Y_{ipn} = \mu + M_i + A_p + MA_{ip} + \varepsilon_{n(ip)} \text{ Model 2}$$

where μ is the true mean; M_i is the effect of $HgCl_2$ concentration (where i = 0–125 μM); A_p is the effect of incubation time

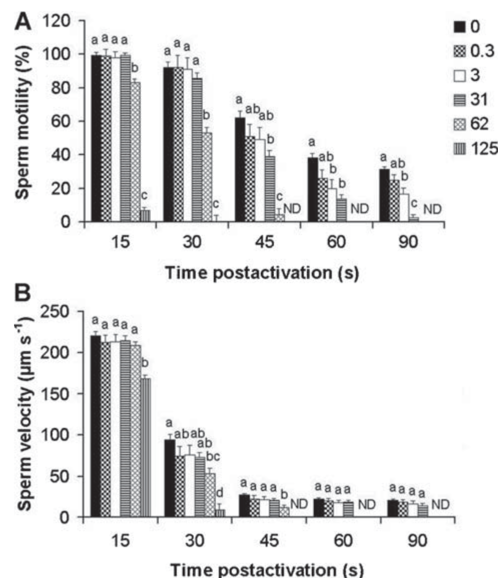

Fig. 1. Sperm motility and velocity in *Perca fluviatilis* after exposure to $HgCl_2$ in an activation medium (AM) (50 mM NaCl, 20 mM Tris, pH 8.5) at ratio 1: 50. Sperm was first diluted in an immobilizing medium (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.5) at a ratio of 1:50. Immediately after predilution, sperm was activated in AM. At each time postactivation, values with different superscripts are significantly different ($p < 0.05, n = 12$). ND = sperm motility or velocity was not assessed because of total suppression of sperm motility by $HgCl_2$.

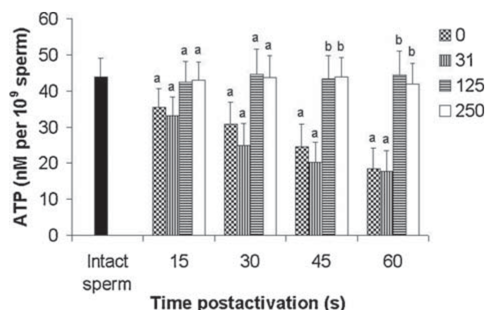

Fig. 2. Adenosine-5′-triphosphate (ATP) content of *Perca fluviatilis* sperm after activation in an activation medium (50 mM NaCl, 20 mM Tris, pH 8.5) at a ratio of 1:50. Sperm was first diluted in an immobilizing medium (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.5) at a ratio of 1: 50 and immediately activated in AM containing different $HgCl_2$ concentrations. At the same time postactivation, values with different superscripts are significantly different ($p < 0.05$, $n = 4$).

(where $p = 0$, 3, 6, and 24 h); MA_{ip} is the $HgCl_2$ concentration × incubation time interaction; and $\varepsilon_{n(ip)}$ is the residual error. When a nonsignificant first-order $HgCl_2$ concentration × incuba-incubation time interaction was detected, the model was re-run with the interaction effect removed, and main effects were then interpreted. In the case of a significant first-order interaction, the model was revised into individual ANOVA models at each incubation time (0, 3, 6, and 24 h) according to model 1 (PROC MIXED; SAS Institute) [25].

The $HgCl_2$ concentration and incubation time were considered fixed in these models, whereas male was considered random and was included as the subject in the RANDOM or REPEATED statement for models 1 and 2, respectively (PROC MIXED; SAS Institute, 2003). The Kenward-Roger procedure was used to approximate the denominator degrees of freedom for all F tests [25]. The repeated statement was used to model the covariance structure within subjects (PROC MIXED; SAS Institute, 2003). Three covariance structures were modeled: compound symmetry (type = cs), autoregressive order 1 (type = ar[1]), and unstructured (type = un). Akaike's (AIC) and Bayesian (BIC) information model-fit criteria were used to assist in final model inference determination [26]. Treatment means were contrasted using the least squares means method (LSMEANS/CL adjust = TUKEY, PROC MIXED; SAS Institute) [25]. Separate ANOVA models were run at each postactivation time.

Experiment 3: Demembranation and reactivation of sperm

Analysis of variance followed by Tukey's test was used to compare sperm motility and velocity in demembranated sperm after activation in AM containing 62 μM with that of control (AM without $HgCl_2$).

RESULTS

Experiment 1: Exposure of nonincubated sperm to $HgCl_2$

Sperm motility and velocity were significantly affected by $HgCl_2$ concentrations in the AM at 15, 30, 45, 60, and 90 s postactivation (Table 1). At 15 and 30 s postactivation, sperm motility significantly decreased at 62 μM (Fig. 1A). At 45 s postactivation, sperm motility was significantly decreased at 31 μM, whereas no motile sperm was observed at 125 μM (Fig. 1A). At 60 and 90 s postactivation, motility significantly decreased at 3 μM (Fig. 1A). No motile sperm was observed in the AM at concentrations higher than 31 μM at 60 and 90 s postactivation (Fig. 1a). At 15 and 30 s postactivation, sperm velocity significantly decreased at 125 and 62 μM, respectively (Fig. 1B). At 45, 60, and 90 s postactivation, a significant decrease in sperm velocity was observed at 62 μM (Fig. 1B). The ATP content of nonactivated (intact) sperm was 44 nM per 10^9 sperm (Fig. 2). Adenosine triphosphate content of cells activated in low (0 and 31 μM) concentrations of $HgCl_2$ were significantly lower compared with sperm cells that had been activated in high (125 and 250 μM) concentrations of $HgCl_2$ at 45 ($F_{3,10} = 4.6$, $p < 0.05$) and 60 s postactivation ($F_{3,11} = 6.1$, $p < 0.01$; Fig. 2). Mercury chloride concentration had no significant effect on ATP content of sperm cells at 15 ($F_{3,8.2} = 1.5$, $p > 0.05$) and 30 s ($F_{3,11} = 2.4$, $p > 0.05$) postactivation (Fig. 2).

Experiment 2: Exposure of incubated sperm to $HgCl_2$

Sperm in $HgCl_2$-free IM activated in AM with $HgCl_2$. For repeated-measures ANOVA, a significant first-order $HgCl_2$ concentration × incubation time interaction effect for sperm motility and velocity was seen at 15, 30, and 45 s postactivation (Table 2). Therefore, at each postactivation time, the models were revised into separate one-way ANOVAs to examine the effect of $HgCl_2$ concentration (0–125 μM) at each incubation time (0–24 h; Fig. 3). A significant effect was detected for all decomposed one-way ANOVA models (Table 3). At 15 and 30 s postactivation, sperm motility at each incubation time significantly decreased at 62 and 31 μM, respectively (Fig. 3A). At 45 s postactivation, motility significantly decreased at 3 μM for the 0-, 3-, and 6-h incubation times, and 31 μM for the 24-h

Table 2. Summary of statistics ($F_{NDF,DDF}$) obtained from two-way analysis of variance (ANOVA) used to compare effects of incubation time, $HgCl_2$ concentrations, and their interactions (incubation time · $HgCl_2$ concentrations) on sperm motility and velocity at different times postactivation[a]

Parameter	Factors	Time postactivation (s) 15	30	45
Sperm motility	Incubation time	$106.2_{3,43}$***	$61.9_{3,42.3}$***	$18.9_{3,40.1}$***
	$HgCl_2$ concentrations	$253.0_{5,43}$***	$233.6_{5,42.1}$***	$255.7_{5,40}$***
	Incubation time · $HgCl_2$ concentrations	$5.7_{15,43}$***	$6.7_{15,42.1}$***	$3.4_{15,40}$**
Sperm velocity	Incubation time	$36.7_{3,45}$***	$18.11_{3,44}$***	$16.6_{3,39.5}$***
	$HgCl_2$ concentrations	$115.1_{5,45}$***	$156.3_{5,44}$***	$114.1_{5,39.5}$***
	Incubation time · $HgCl_2$ concentrations	$11.3_{15,45}$***	$4.1_{15,44}$***	$3.4_{15,39.5}$**

[a] In this experiment, sperm was incubated in an immobilizing medium without $HgCl_2$ and then activated in an activation medium containing different $HgCl_2$ concentrations.
** $p < 0.001$.
*** $p < 0.0001$.

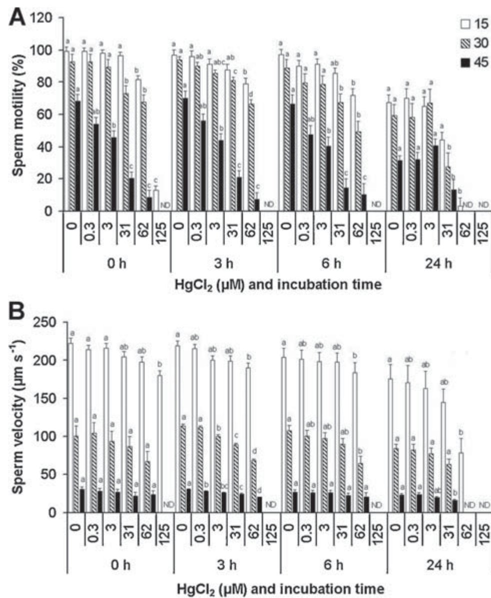

Fig. 3. Motility (**A**) and velocity (**B**) of incubated sperm of *Perca fluviatilis* in an immobilizing medium (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.5) without $HgCl_2$ after activation in an activation medium (50 mM NaCl, 20 mM Tris, pH 8.5) containing different $HgCl_2$ concentrations at 15, 30, and 45 s postactivation. At each time postactivation and incubation time, values with different superscripts are significantly different ($p < 0.05$) ($n = 4$). ND = sperm motility was not assessed because of total suppression of sperm motility by $HgCl_2$.

incubation time (Fig. 3A). At 15 s postactivation, velocity significantly decreased at 125 µM for the 0-h incubation time, and 62 µM for the 3-, 6-, and 24-h incubation times (Fig. 3B). At 30 s postactivation, sperm velocity significantly decreased at 3 and 62 µM $HgCl_2$ at 3- and 6-h incubation times, respectively (Fig. 3B). At 45 s postactivation, a significant decrease of sperm velocity was observed at 3 and 31 µM $HgCl_2$ at 3- and 24-h incubation, respectively (Fig. 3B).

Sperm in IM with $HgCl_2$ activated in AM with $HgCl_2$. For repeated-measures ANOVAs, a significant first-order $HgCl_2$ concentration × incubation time interaction effect for sperm motility was seen at 15, 30, and 45 s postactivation (Table 4). Therefore, at each activation time, the models were revised into separate one-way ANOVAs to examine the effect of $HgCl_2$ concentration (0–125 µM) at each incubation time (0–24 h; Fig. 4A). A significant effect was detected for all of these decomposed one-way ANOVA models (Table 3). At 15, 30, and 45 s postactivation, no motile sperm were found when $HgCl_2$ concentrations were greater than 3 µM in both the IM and AM (Fig. 4A). At 15 s postactivation, motility did not differ in a range of 0 to 3 µM for the 0-h incubation, but it significantly decreased at 3 µM for the 3- and 6-h incubation times (Fig. 4A). At 30 s postactivation, motility did not differ in range of 0 to 3 µM for the 0-h incubation, but it significantly decreased at 3 µM for the 3- and 6-h incubation times, and 0.3 µM for the 24-h incubation time (Fig. 4A). At 45 s postactivation, a significant difference of sperm motility was observed at 0.3 and 3 µM $HgCl_2$ after 0-, 3-, and 6-h incubations (Fig. 4A).

For repeated-measures ANOVAs, a significant first-order $HgCl_2$ concentration × incubation time interaction effect for sperm velocity was seen at 15 s postactivation (Table 4). Therefore, at 15 s postactivation, the model was revised into separate one-way ANOVAs to examine the effect of $HgCl_2$ concentration (0–125 µM) at each incubation time (0–24 h; Fig. 4B). A significant effect was detected for all decomposed one-way ANOVA models (Table 3). Velocity decreased at 3 µM for the 0-, 3-, 6-, and 24-h incubation times (Fig. 4B). The first-order $HgCl_2$ concentration × incubation time interaction effect was not significant for sperm velocity at 30 and 45 s postactivation (Table 4). The models were therefore re-run with the $HgCl_2$ concentration × incubation time interaction effect removed, and main effects were interpreted. At 30 s postactivation, $HgCl_2$ concentration ($F_{5,59} = 136.7$, $p < 0.0001$) and incubation time ($F_{3,59.2} = 3.4$, $p < 0.05$) had an effect on velocity. At 45 s postactivation, $HgCl_2$ concentration had an effect on velocity ($F_{5,60} = 169.35$, $p < 0.0001$). Conversely, incubation time had no effect on sperm velocity ($F_{3,60} = 2.51$, $p > 0.05$).

For repeated-measures ANOVA, a significant first-order $HgCl_2$ concentration × incubation time interaction effect for ATP content ($F_{10,61} = 11.3$, $p < 0.0001$) was seen. Therefore, the models were revised into separate repeated-measure ANOVAs to examine the effects of $HgCl_2$ (0–125 µM) at each

Table 3. Summary of statistics ($F_{NDF,DDF}$) obtained from one-way analysis of variance (ANOVA) used to compare effects of $HgCl_2$ concentrations on sperm motility and velocity at different times postactivation and incubation time[a]

Source	Sperm parameter	Time post sperm activation (s)	Incubation time (h)			
			0	3	6	24
IM without $HgCl_2$, AM with $HgCl_2$	Motility	15	$201.7_{5,12}^{***}$	$284.4_{5,10}^{***}$	$164.0_{5,9.2}^{***}$	$49.8_{5,10}^{***}$
		30	$75.0_{5,10}^{***}$	$543.2_{5,10}^{***}$	$45.8_{5,9.2}^{***}$	$19.3_{5,7.8}^{**}$
		45	$60.0_{5,9.2}^{***}$	$63.2_{5,10}^{***}$	$27.7_{5,9.1}^{***}$	$27.3_{5,6.2}^{**}$
	Velocity	15	$8.6_{5,10}^{*}$	$705.7_{5,10}^{***}$	$363.8_{5,9}^{***}$	$10.9_{5,10}^{**}$
		30	$31.5_{5,10}^{***}$	$797.8_{5,12}^{***}$	$77.3_{5,9}^{***}$	$105.4_{5,7.5}^{***}$
		45	$23.3_{5,8}^{***}$	$526.8_{5,10}^{***}$	$20.3_{5,8.1}^{**}$	$97.1_{5,8.2}^{***}$
IM and AM with $HgCl_2$	Motility	15	$123.6_{5,10}^{***}$	$16.9_{5,10}^{***}$	$214.8_{5,8.1}^{***}$	$30.1_{5,11}^{***}$
		30	$140.9_{5,12}^{***}$	$90.2_{5,12}^{***}$	$38.2_{5,12}^{***}$	$16.1_{5,9}^{**}$
		45	$175.3_{5,10}^{***}$	$42.6_{5,12}^{***}$	$47.5_{5,10}^{***}$	$33,\ _{5,10}^{***}$
	Velocity	15	$914.8_{5,10}^{***}$	$410.6_{5,12}^{***}$	$630.7_{5,9.2}^{***}$	$467.5_{5,9.3}^{***}$

[a] IM = immobilizing medium; AM = activation medium.
[*] $p < 0.01$.
[**] $p < 0.001$.
[***] $p < 0.0001$.

Table 4. Summary of statistics ($F_{NDF,DDF}$) obtained from two-way analysis of variance (ANOVA) used to compare effects of incubation time, $HgCl_2$ concentrations and their interactions (incubation time · $HgCl_2$ concentrations) on sperm motility and velocity at different times postactivation[a]

Parameter	Factors	Time postactivation (s)		
		15	30	45
Sperm motility	Incubation time	$13.2_{3,43.1}$***	$15.6_{3,45.1}$***	$14.2_{3,44.1}$***
	$HgCl_2$ concentrations	$171.2_{5,43.1}$***	$192.3_{5,45.1}$***	$202.9_{5,44.1}$***
	Incubation time · $HgCl_2$ concentrations	$4.0_{15,43}$**	$5.1_{15,45.1}$***	$4.0_{15,44.1}$**
Sperm velocity	Incubation time	$14.1_{3,44.3}$***	$4.5_{3,44.1}$*	$2.7_{3,45}$
	$HgCl_2$ concentrations	$188.6_{5,44.2}$***	$163.7_{5,44}$***	$160.8_{5,45}$***
	Incubation time · $HgCl_2$ concentrations	$3.2_{15,44.2}$***	$1.8_{15,44}$	$0.8_{15,45}$

[a] In this experiment, both immobilizing and activation media contains the same concentrations of $HgCl_2$.
* $p < 0.01$.
** $p < 0.001$.
*** $p < 0.0001$.

incubation time (0, 3, 6, and 24 h) (Fig. 5). The ATP content did not differ between $HgCl_2$ concentrations at 0 h after incubation ($F_{5,16} = 1.13$, $p > 0.05$). The ATP content was affected by $HgCl_2$ at 3 h ($F_{5,13.2} = 11.2$, $p < 0.001$), 6 h ($F_{5,13.2} = 12.8$, $p < 0.0001$), and 24 h ($F_{5,13} = 5.1$, $p < 0.01$) after incubation. The ATP content significantly decreased at 3 μM when sperm was incubated for 3 or 6 h and at 62 μM when sperm was incubated for 24 h (Fig. 5).

Experiment 3: Demembranation and activation of sperm

Sperm motility of demembranated sperm was not activated at 125 μM $HgCl_2$. Sperm motility (from 81.4 ± 1.3 to

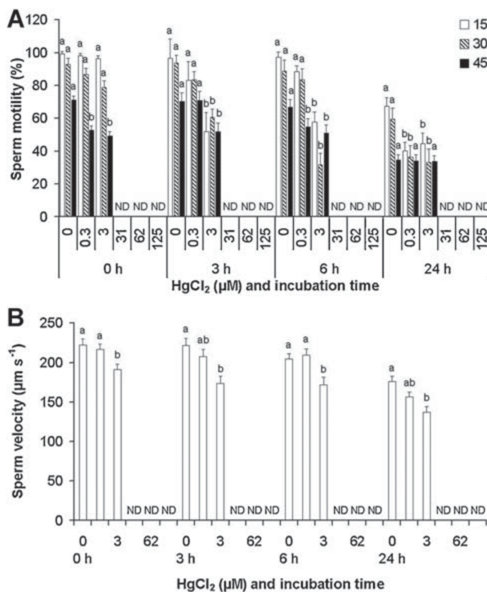

Fig. 4. Motility (**A**) and velocity (**B**) of incubated sperm of *Perca fluviatilis* in immobilizing medium (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.5) containing different concentrations of $HgCl_2$ after activation in an activation medium (50 mM NaCl, 20 mM Tris, pH 8.5) with the same concentration of $HgCl_2$ concentrations at 15, 30, and 45 s postactivation. At each time postactivation and incubation time, values with different superscripts are significantly different ($p < 0.05$) ($n = 4$). No interaction was seen between $HgCl_2$ and incubation time for 30 and 45 s postactivation in terms of sperm velocity. ND = sperm motility was not assessed because of total suppression of sperm motility by $HgCl_2$.

$61.9 \pm 1.2\%$; $F_{4,5} = 25.7$, $p < 0.01$) and velocity (from 88.1 ± 3.1 to 72.4 ± 2.3 μm/s; $F_{4,5} = 25.7$, $p < 0.0001$) significantly decreased after activation of demembranated sperm in 62 μM $HgCl_2$.

Effect of $HgCl_2$ on sperm morphology

Perch sperm heads have an ovoid shape and contain the nucleus with heterogeneous, condensed, and granular chromatin material. Proximal and distal centrioles with nine triplets of peripheral microtubules, as well as one to two mitochondria, were located in the midpiece. The proximal centriole was closely adjacent to the nuclear envelope and was located at a right angle to the distal centriole, which serves as the basal body of the flagellum. We observed a cytoplasmic channel, which is formed by an invagination of the plasmalemma. The flagellum is composed of a 9 + 2 structure of microtubules and has paired lateral ribbons (Figs. 6 and 7; $HgCl_2$ 0 μM and incubation time 0 h).

Scanning electron microscopy and transmission electron microscopy showed no damage of sperm in our control groups during incubation (Figs. 6 and 7). Using scanning electron microscopy, agglutinated or agglomerated spermatozoa were observed at higher than 31 μM $HgCl_2$, in a curling shape from 3 h after incubation (Fig. 6). The same forms of spermatozoa were observed from 3 h after incubation at $HgCl_2$ higher than 62 μM, and the flagellum of sperm sustained injuries; for example, the plasma membrane was impaired and cut (Fig. 6). Transmission electron microscopy showed that the plasma membrane was swollen, but axoneme was not disrupted in control or in low concentrations of $HgCl_2$ (0.3 μM) (Fig. 7). At 3 μM $HgCl_2$, sperm morphology was affected at 3 to 6 h after incubation by injuries in the plasma membrane and impairing of mitochondria, whereas the structure of the axoneme was still not damaged. After 24 h incubation, the axoneme structure and mitochondria were damaged. The intensity of damage depended on incubation time. For example, sperm incubated at 62 μM $HgCl_2$ showed damage of plasma membrane at 0 h after incubation, whereas axonemal structure was disrupted at 24 h after incubation (Fig. 7).

DISCUSSION

Bioaccumulation of Hg in fish tissue, such as the gonad and liver, may lead to levels 100 to 10,000 times higher than the Hg concentrations in their surrounding environment [14]. Mercury levels can, however, be much higher in waters contaminated by point-source discharges from chloralkali plants and mining sites, with levels in the range of 0.15 to

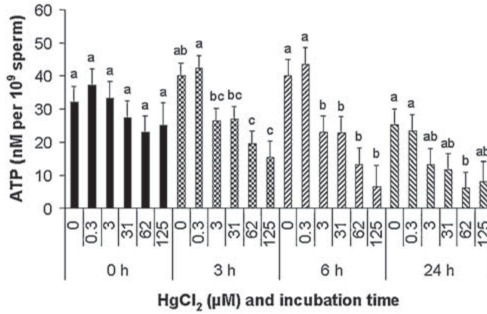

Fig. 5. Adenosine-5′-triphosphate (ATP) content of sperm of *Perca fluviatilis* after incubation in an immobilizing medium (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.5) containing different concentrations of $HgCl_2$. At each incubation time, values with different superscripts are significantly different ($p < 0.05$) ($n = 4$).

0.70 mg/L (0.55–2.58 µM) Hg [14]. These data suggest that environmentally realistic levels of Hg may be having an impact on the reproduction of fish in the wild [27]. We explored the mechanisms of action of Hg on sperm performance by using European perch as our model organism. Perch sperm were exposed to Hg in two different ways: instant exposure, which mimics the effects of Hg on sperm released into a polluted aquatic environment, and during an incubation period in a non-mobility extender, which mimics effects that occur during storage of sperm (for up to 24 h) in the reproductive system [1].

In experiment 1, we observed a decrease in sperm performance after activation in an AM containing $HgCl_2$. Such a decrease has been observed in previous studies and ultimately leads to a decrease in fertilization success [12–16]. In the literature, effective concentrations of $HgCl_2$ have been shown to differ among aquatic species in a dose- or exposure time–dependent manner [12–15]. These species-specific tolerances to $HgCl_2$ may be related to sperm function and design. Alternatively, they may be species-specific mechanistic responses. Currently, available literature to explain these observed differences is lacking. Dietrich et al. [15] proposed seminal plasma

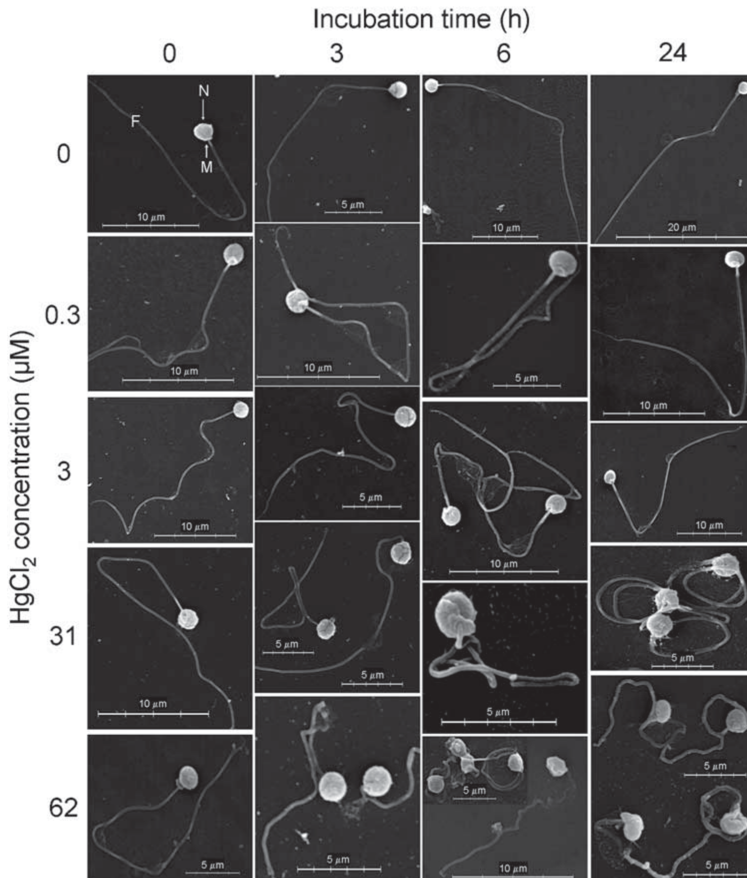

Fig. 6. Changes of morphology of sperm of *Perca fluviatilis* after incubation in $HgCl_2$ immobilizing medium (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.0) using scanning electron microscopy. N, M, and F are nucleus, midpiece, and flagellum, respectively.

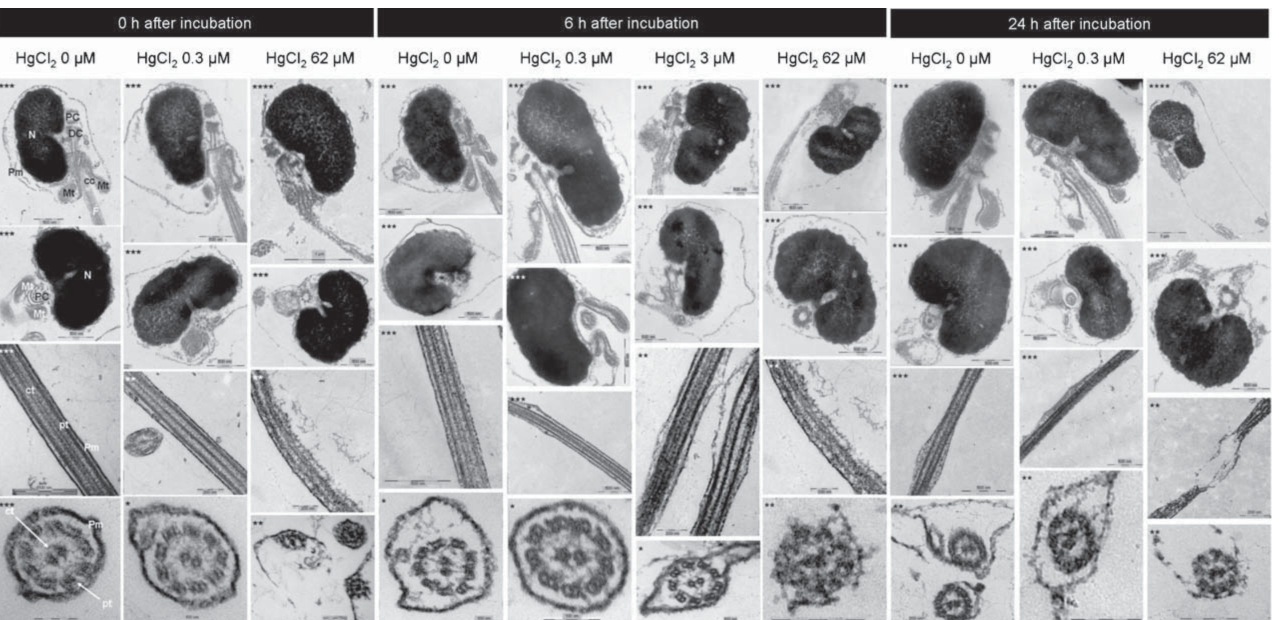

Fig. 7. Ultrastructural changes of sperm of *Perca fluviatilis* after incubation in $HgCl_2$, an immobilizing medium (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.0), using transmission electron microscopy. N = nucleus; PC = proximal centriole; DC = distal centriole; Pm = plasma membrane; Mt = mitochondria; cc = cytoplasmic channel; pt = peripheral microtubules; ct = central microtubules. Scale bars for micrographs with *, **, ***, and **** are 100 nm, 200 nm, 500 nm, and 1 μm, respectively.

characteristics as a possible cause, because incubated sperm in the seminal plasma containing Hg presented better motility compared with incubated sperm in an IM. Wojtczak et al. [28] reported transferrin as a major seminal plasma protein in common carp (*Cyprinus carpio*). This protein is known to be an important binding protein for heavy metals in rainbow trout blood [29]. Therefore, further studies on seminal plasma proteins are required to understand mechanisms of protection of sperm against Hg compounds or other endocrine disruptors.

In experiment 2, stronger effects of $HgCl_2$ were observed when sperm were incubated in the IM with $HgCl_2$ compared with the effects on incubated sperm in the IM without $HgCl_2$. Van Look and Kime [14] also observed a decrease in sperm performance when sperm were incubated in an IM with $HgCl_2$ for 24 h. Motility and velocity of incubated sperm decreased at 0.37 μM $HgCl_2$ compared with 3.68 μM $HgCl_2$ for nonincubated sperm [14].

In experiment 1, ATP content of sperm did not change after activation at high $HgCl_2$ concentrations (>125 μM $HgCl_2$). This means that enough energy was available, in the form of readily available ATP, for axonemal beating, but the structure of the sperm was damaged. This structural damage was confirmed by using demembranated sperm (experiment 3). In this experiment, we were able to pinpoint the sperm flagella as a site of action of $HgCl_2$. In previous studies, flagella damage has been shown to adversely affect sperm movement [16,30]. For example, Billard et al. [30] analyzed flagella morphology on fresh and cryopreserved sperm of the Siberian sturgeon (*Acipenser baerii*). Their results showed a reduction in flagellum beating efficiencies, and distinct flagellum abnormalities (e.g., nodules, loop located at the distal tip), which they speculated decreased overall motility in cryopreserved samples. Abascal et al. [16] observed that more than 20 μM $HgCl_2$ totally suppressed motility of demembranated spermatozoa of sea bass after activation in an AM containing 1 mM ATP. Van Look and Kime [14] observed significantly shorter flagellum length when goldfish sperm were exposed to 368 μM $HgCl_2$ in an instant exposure experiment and to 0.0037 μM in incubated exposure for 24 h. This observation could be attributable to agglutination or agglomeration of sperm cells or disruption of flagella, which we observed at high $HgCl_2$ concentrations (>31 μM).

Using electron microscopy, we also observed damage to the sperm plasma membrane and axoneme. Therefore, we consider the plasma membrane and axoneme as sites of action of $HgCl_2$. Structural damages were more distinct by increasing $HgCl_2$ concentration or incubation time. Sperm morphology and fine structure was conserved in our control and low $HgCl_2$ concentrations (i.e., 0.3 μM). More damage was observed at concentrations greater than 3 μM. Coiled flagella and kinks in the midpiece and flagellum regions have been reported in rat and long-tailed macaque when sperm were exposed to 36.8 and 3.68 μM methyl Hg, respectively, in vitro [31,32].

Sperm motility mainly depends on ATP stores from a prior ejaculation [33]. When sperm was incubated in the IM containing $HgCl_2$ for longer than 3 h, ATP content of sperm in the IM was greater than 3 μM, and $HgCl_2$ was lower than that of control or less than 3 μM $HgCl_2$, which might be caused by damage of mitochondria as observed by transmission electron microscopy. In the present study, damage of the midpiece was observed at 3 or 62 μM $HgCl_2$ after a 24- or 6-h incubation, respectively. Perhaps this could explain the lower sperm velocity at 3 μM $HgCl_2$, as well as the morphological damage we observed. Rao and Sharma [4] reported a reduction of ATPase level in mice sperm treated with $HgCl_2$. Sperm activation is an ATP-dependent mechanism. Adenosine-5′-triphosphate provides the source of energy for dynein ATPase activity, which hydrolyzes ATP to produce flagellar beating [34].

Fish spermatozoa are immotile in the seminal plasma, and a hypoosmotic signal is necessary for triggering sperm activation in freshwater fish species such as perch [33,35,36]. On receiving the osmotic signal, ion channels and aquaporins regulate exchange of ions and water through the plasma membrane, respectively, which lead to the initiation of sperm motility [35–37]. Zilli et al. [37] observed that $HgCl_2$ at 10 μM completely inhibited sperm activation in gilthead sea bream (*Sparus aurata*). Their study showed the existence of aquaporins in fish spermatozoa that are detected at the surface of the spermatozoa, both head and flagella. Therefore, they suggested the presence of Hg-sensitive aquaporins that regulate sperm activation. Roles of aquaporins remained to be investigated in freshwater species such as perch. Mohamed et al. [32] showed that sulfhydryl groups in the membrane, head, midpiece, and tail of the sperm are sites of Hg binding. Taken together, damaging of plasma membrane by $HgCl_2$ led to disruption of its physiological role in sperm activation.

In conclusion, $HgCl_2$ acts on different sites of sperm, such as the plasma membrane, axoneme, and midpiece (mitochondria). Incubation of sperm enhances $HgCl_2$ effects by decreasing stored ATP and increasing morphological damage to sperm during the activation period.

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In vitro effects of Bisphenol A on sperm motility characteristics in *Perca fluviatilis* L. (Percidae; Teleostei)

By A. Hatef, S. M. H. Alavi, Z. Linhartova, M. Rodina, T. Policar and O. Linhart

South Bohemia Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Zatisi, Vodnany, Czech Republic

Summary

In the present study, the effects of Bisphenol A (BPA) on sperm motility and velocity were investigated in perch (*Perca fluviatilis* L.). Sperm of five mature males was separately collected in a syringe. The sperm samples were diluted in an immobilizing solution containing 180 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 2.4 mM $NaHCO_3$, pH 8.0 (340 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : IS). The sperm motility was triggered in an activation solution composed of 50 mM NaCl, 20 mM Tris, pH 8.5 containing different concentrations of BPA (2, 1.5, 1.25, 1.0, 0.5, 0.25, 0.125 and 0.0 mM) at ratio 1 : 50 (sperm : AS). At 15 s post-activation, sperm motility and velocity decreased significantly in activation medium containing 1.5 and 1 mM BPA, respectively. The motility of sperm was totally inhibited in 2 mM of BPA. Sperm motility duration decreased with increasing BPA concentrations. In the control, 25.3% of spermatozoa were still motile at 90 s post-activation, while only 1.3% was in 0.12 mM. However, sperm velocity was higher in activation medium containing 0.12 mM BPA at 15 s post-activation. There was no significant difference between the control and DMSO test group, the latter being used for dissolving BPA. The results of this study indicate that BPA decreases both motility and velocity of exposed sperm, *in vitro*. BPA may damage the flagella as 'C' shape of flagella was observed, once motility period of sperm cells are finished.

Introduction

Endocrine disrupting chemicals (EDCs) are a large group of synthetic and natural occurring agents that interfere with the normal endocrine functions (Kime, 1998). The US-Environmental Protection Agency (EPA) has defined EDC as 'an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental process' (Kavlock et al., 1996). EDCs change the endocrine system through receptor-mediated or receptor-independent mechanisms (Gregory et al., 2008), which lead to intersexes, suppress spermatogenesis, or decrease reproductive success (Sumpter and Jobling, 1995; Kime, 1998). Decrease in reproductive ability may results from altered sperm quality (such as decreased sperm production or reduced sperm motility).

Bisphenol A (BPA) is one of the highest volume chemicals produced worldwide, with over six billion pounds produced each year (Vandenberg et al., 2009). BPA is building block of polycarbonate plastic. BPA leaches from polycarbonate baby bottles, and reusable water bottles. Epoxy resins used to protect food cans are also synthesized by the condensation of BPA with epichlorhydrin to create BPA diglycidyl ether (Richter et al., 2007; Vandenberg et al., 2009). BPA act as an estrogen mimic, directly with estrogen receptor (ER) and with plasma sex-steroid binding protein (Tollefsen et al., 2004). The affinity of BPA for the ERs is approximately 10 000-fold weaker than that of estradiol (Gregory et al., 2008). In addition to estrogenic activity of BPA, there is some evidence that BPA inhibits or decrease testicular steroidogenesis at low exposure level (Sohoni and Sumpter, 1998; Akingbemi et al., 2004). It was shown that BPA alters sperm concentration in guppies (*Poecilia reticulata*) (Haubruge et al., 2000) and brown trout (*salmo trutta fario*) at the beginning of the spawning period (Lahnsteiner et al., 2005b). It can also reduce numbers of spermatogenic cysts and induce intersex in common carp (*Cyprinus carpio* L.) (Mandich et al., 2007). Increase of serum vitellogenin concentrations have been reported in male Atlantic cod (*Gadus morhua*) (Larsen et al., 2006), medaka (*Oryzias latipes*) (Tabata et al., 2001; Kang et al., 2002) and fathead minnow (*Pimephales promelas*) (Sohoni et al., 2001) exposed to BPA. Lahnsteiner et al. (2005b) studied the *in vivo* effects of BPA on sperm characteristic in brown trout. However, no information is available about *in vitro* effects of BPA on sperm motility.

In the present study, instant effects of BPA on sperm motility and velocity were studied in Eurasian perch, *Perca fluviatilis* L. (Percidae, Teleostei) using a Computer Assisted Sperm Analysis (CASA) system, *in vitro*. Spermatozoa concentration of perch was reported 29×10^9 spz ml^{-1} (Alavi et al., 2007). The spermatozoa are immotile in the seminal fluid due to osmolality and a hypo-osmotic shock is necessary for triggering of initiation of sperm motility (Alavi et al., 2007, 2010). Duration of sperm motility is very short (< 1 min in freshwater). Spermatozoa velocity, percentage of motility and beat frequency decrease rapidly after sperm activation that depends on the osmolality of the activation medium (Alavi et al., 2007, 2010). Osmolality higher than 300 mOsmol kg^{-1} totally suppress sperm activation. The means of seminal plasma osmolality (mOsmol kg^{-1}), sodium, chloride, potassium and calcium ions concentrations (mM) are measured as 298, 131, 107, 11 and 2.4 respectively (Alavi et al., 2007).

Materials and methods

Broodfish and collection of semen

Sperm was collected from five mature males of perch (total length: 205–230 mm, and body weight: 110–162 g) in the second half of March, 2009. The males were anesthetized

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before stripping in clove oil (33 mg L^{-1}). The sperm was collected by a gentle abdominal massage from the anterior portion of the testis towards the genital papilla and collected with plastic syringes to avoid sperm contamination by urine, mucus and blood cells. Samples of sperm were stored at 2–4°C and transferred to the laboratory of reproductive physiology in fish for further analysis. Spermatozoa concentration, spermatocrit and osmolality of the collected samples were between 54.3 and 68.59×10^9 spz ml^{-1}, 66–70% and 300–306 mOsmol kg^{-1}.

Sperm dilution and motility assessment

After collection of sperm, a two-step dilution was used to evaluate sperm motility. Firstly, sperm was diluted in an immobilizing solution (IS) containing 180 mm NaCl, 2.68 mm KCl, 1.36 mm $CaCl_2$, 2.38 mm $NaHCO_3$, pH 8.0 (340 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : IS). Then, the sperm was activated in activation solution (AS) containing 50 mm NaCl, 20 mm Tris, pH 8.5 (110 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : AS). To test the effects of BPA, dissolved BPA in DMSO was added to AS at 2, 1.5, 1.25, 1.0, 0.5, 0.25, 0.12 mm. DMSO was < 0.1% after adding BPA in activation medium. Therefore, effect of DMSO 0.1% on sperm motility was studied as control. To avoid stickiness of sperm into the glass slide, 0.1% BSA was only added to the activation solution before sperm activation. Preliminary observations did not show any difference between activation solution containing BSA and activation solution without BSA at different concentration of BPA in terms of sperm motility (viability). In both conditions, flagellar beating was observed, but only forward motions of spermatozoa were observed in activation solution containing BSA. In activation solution without BSA, forward motions of spermatozoa inhibited due to stickiness of sperm into the glass slide.

Sperm motility was recorded using a three CCD video camera (SONY DXC-970MD, Japan) mounted on a dark-field microscope (Olympus BX50, Japan) equipped with a stroboscopic lamp at frequency adjusted to 50 Hz. A micro image analyzer (Olympus Micro Image 4.0.1 for Windows – CASA) was used to measure percentage of sperm motility (%) and sperm velocity ($\mu m\ s^{-1}$) from five successive video images, which shows positions of sperm heads. Five video frames were captured from a DVD-recorder (SONY DVO-1000 MD, Japan) and accumulated (overlapped) using CASA in real time post-activation. Figure 1 shows successive frames of sperm movement in *P. fluviatilis* at 13 s post-activation and the final output of the CASA from overlapping the five captured frames that was used for measuring the sperm parameters. The out put frame is showing positions of head of

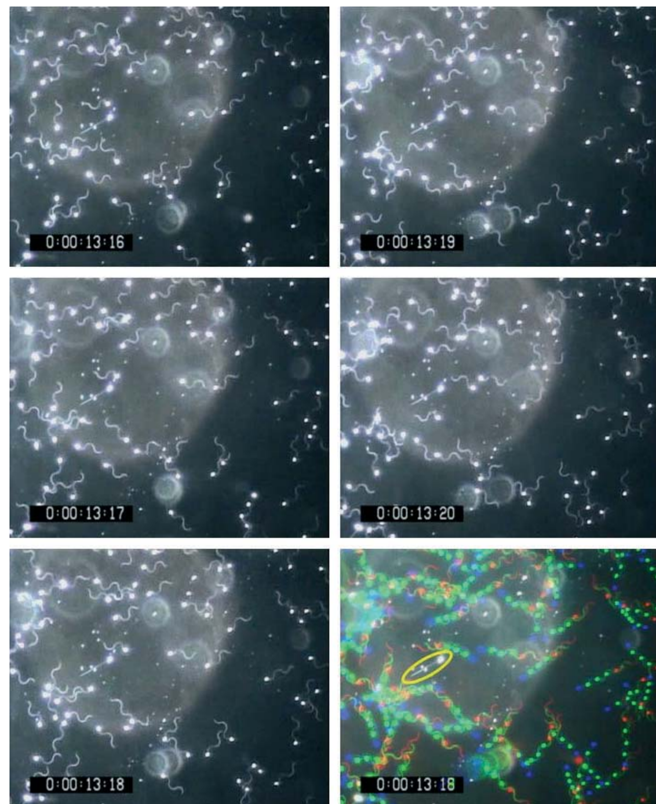

Fig. 1. Successive frames of spermatozoa movement in *Perca fluviatils* identified by the time (bottom left, in this case at 13 s post-activation). The out put frame from overlapping of five captured frames is showing five positions of heads of motile spermatozoa in red-green-green-green-blue, while the immotile sperm cell(s) is in white color (in this case the spermatozoon in yellow circle). The percentage of motile spermatozoa was calculated by counting of red or blue spots versus the number of white spots. To measure the sperm velocity, distance of five head positions (distance between red and blue spots) were measured and divided into time spent for moving such distance

motile spermatozoa in five spots (red-green-green-green-blue), while the immotile spermatozoa are shown in white color. Therefore, the percentage of motile spermatozoa was calculated by counting of red or blue spots versus the number of white spots. To measure the sperm velocity, distance of five head positions (distance between red and blue spots) were measured and divided into time spent for moving such distance. In this study, the sperm velocity shows data of only motile spermatozoa (approximately 50–60 spermatozoa per each treatment). In each treatment, sperm motility was assessed three times per each male.

Data analysis

Data presented are mean ± standard error of mean (SE). Mean of data from three analyses per each treatment were measured and used for statistical analysis. Before analysis of data by ANOVA, Kolmogorov–Smirnov 's and Levene 's tests were used for normality of data distribution and homogeneity of variances. Then, statistical comparisons were made based on sperm motility and velocity as dependent variables and BPA concentration as independent variable at each time post sperm activation following Duncan's test (SPSS 10.0).

Results

Significant effect of BPA on sperm motility was observed at each time post-activation (df = 4, P < 0.001, F = 66.87, 67.04, 49.40, 33.17 and 60.11 at 15, 30, 45, 60 and 90 s post-activation, respectively). The motility of sperm was totally inhibited in 2 mM BPA (Fig. 2). The duration of sperm motility decreased with increasing BPA concentrations. In control, 25.3% of spermatozoa were motile at 90 s post-activation, while only 1.3% was motile in 0.12 mM. At 15 s post-activation, the percentage of sperm motility significantly decreased after activation in 1.5 mM BPA. The percentage of motile spermatozoa did not differ significantly between control and sperm exposed to lower than 1.25 mM BPA at 15 s post-activation, but decreased significantly in 1.0 mM BPA at 30 and in 0.5 mM BPA at 45 s post-activation. Closer to the end of the motility period, sperm motility was significantly low in very low concentrations of BPA (0.25 and 0.12 mM at 60 and 90 s post-activation, respectively). There was no significant difference between control and activation solution containing 0.1% DMS in terms of sperm motility (Fig. 2).

At 15 s post-activation, sperm velocity was significantly influenced by BPA concentrations and decreased significantly in 1 mM BPA (df = 4, F = 151.10, P < 0.001). Sperm velocity was higher in 0.12 mM than in control and DMSO groups (Fig. 3). BPA concentrations also affected sperm velocity at 30 and 45 s post-activation (df = 4, F = 29.06 and 17.17, P < 0.001). At 30 s post-activation, the highest sperm velocity was observed in activation solution containing 0.12 mM BPA. Sperm velocity in BPA 0.25–1.25 mM were higher than control and DMSO (Fig. 3). BPA concentration showed significant effect on sperm velocity at 45 s post-activation (df = 4, F = 15.17, P < 0.01). The highest sperm velocity was observed in activation solution containing 0.12–1.25 mM BPA. Sperm velocity was also influenced by BPA concentrations at 60 (df = 4, F = 1.58, P < 0.05) and 90 s post-activation (df = 4, F = 1.16, P < 0.05). Significant differences were not observed between the control, DMSO and BPA concentrations (0.12–0.25 mM), in which the spermatozoa were motile. There was no significant difference in terms of sperm velocity between control and DMSO, which was used for dissolving BPA (Fig. 3).

Figure 4 shows sperm motility of *P. fluviatilis* after activation in control or different BPA concentrations at different time post-activation. Interestingly, a 'C' shape of flagella was observed at 1.25 mM BPA (at 60 s post-activation) and at 1.5 mM BPA (at 30 s post-activation), when the motility period is finished. In the control, flagellum of sperm is in straight position once the motility period is finished at 90 s post-activation.

Discussion

The present study showed that BPA affect on sperm motility and velocity of perch, *in vitro*. LC50 was estimated at 1.37 mM BPA (Fig. 2). BPA may damage the flagella as different form

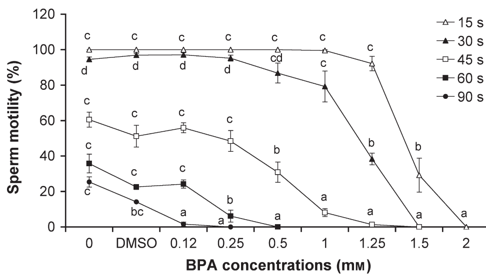

Fig. 2. *In vitro* effects of bisphenol A (BPA) on sperm motility in *Perca fluviatilis*. Sperm of five males were separately diluted in an immobilizing solution containing 180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.0 (340 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : IS). Immediately after dilution, the sperm motility was activated in activation solution containing 50 mM NaCl, 20 mM Tris, pH 8.5 (110 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : AS). BPA was only added into activation solution (DMSO concentration was lower than 0.1% after adding BPA in activation solution). Effect of DMSO 0.1% on sperm motility was also studied. At the same time post-activation, values (mean ± standard error of mean) with the same letters are not significantly different (P > 0.05)

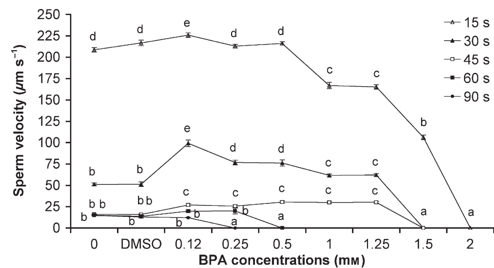

Fig. 3. *In vitro* effects of bisphenol A (BPA) on sperm velocity in *Perca fluviatilis*. Sperm of five males were separately diluted in an immobilizing solution containing 180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.0 (340 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : IS). Immediately after dilution, the sperm motility was activated in activation solution containing 50 mM NaCl, 20 mM Tris, pH 8.5 (110 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : AS). BPA was only added into activation solution (DMSO concentration was lower than 0.1% after adding BPA in activation solution). Effect of DMSO 0.1% on sperm motility was also studied. At the same time post-activation, values (mean ± standard error of mean) with the same letters are not significantly different (P > 0.05)

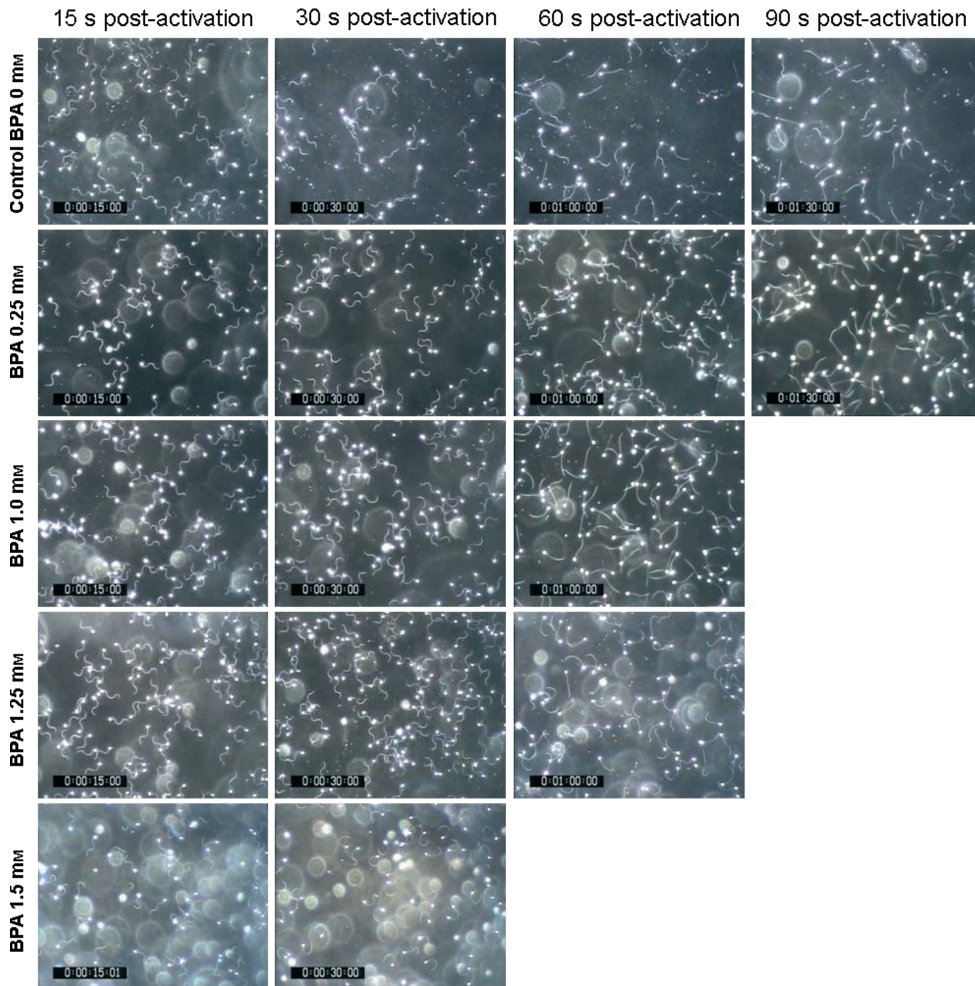

Fig. 4. *In vitro* effects of BPA on sperm flagella in *Perca fluviatilis*. Once the motility period of sperm is finished after activation, structure of flagella become 'C' shape in sperm that has been exposed to high concentration of BPA (> 1.25 mM) compared to straight structure of sperm in control. In all cased, sperm was firstly diluted in an immobilizing solution containing 180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 2.38 mM $NaHCO_3$, pH 8.0 (340 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : IS). The sperm motility was activated in activation solution containing 50 mM NaCl, 20 mM Tris, pH 8.5 (110 mOsmol kg^{-1}) at ratio 1 : 50 (sperm : AS). BPA was only added to activation solution

of flagella are observed once motility period of sperm cells are finished (Fig. 4). However, it is not well know if BPA damage the plasma membrane or axoneme. Plasma membrane plays an important role in the initiation of sperm motility due to hypo-osmotic signals (Alavi et al., 2007). Thomas and Doughty (2004) observed that nonestrogenic as well as estrogenic organic compounds could interfere with a rapid nongenomic progestin action to upregulate sperm motility in Atlantic croaker. Most of these chemicals (such as BPA, estradiol, *p,p′*-DDT, *o,p′*-DDE, naphthalane, zearalenone) completely blocked the hormonal response to 17,20β,21 – trihydroxy-4-pregnen-3-one (20β-S) at 0.1 μM, but none of them caused a decrease in percentage of motile sperm below control levels. Some of these chemicals can bind to the sperm membrane progestin receptor such as *o,p′*-DDE and zearalenone (Thomas et al., 1998) suggesting that their mechanisms of disruption involve binding to the 20β-S receptor. In addition, the effect of BPA on sperm energetics (ATP content and consumption during sperm activation) is another option that could be considered in further studies. ATP is the most energetic source required for axonemal beating in fish sperm (Perchec-Poupard et al., 1998; Rurangwa et al., 2002). Lahnsteiner et al. (2005b) studied the *in vivo* effects of BPA on sperm characteristics in brown trout during reproductive season. They found that sperm motility and velocity were lower in males exposed to 1.75, 2.40 and 5.0 μg L^{-1} BPA for 3 weeks at the beginning

and the middle of the reproductive season, but no effects was observed at the end of reproductive season. Our recent study on goldfish (*Carassius carassius* L.) showed decrease of sperm motility after exposing fish to BPA higher than 1 μg L^{-1} for 1 month that might be related to anti-androgenic activity of BPA (Hatef, A., Alavi, S.M.H., Abdalfatah, A., Fontaine, P., Linhart, O., unpubl. data). There are many studies showing estrogenic activity of BPA through direct ER binding or indirect actions of endogenous E2 (Crain et al., 2007). BPA enhances VTG mRNA expression and increase the VTG concentration in serum (Tabata et al., 2001; Yamaguchi et al., 2005; Huang et al., 2010). BPA can also act through sex steroid binding proteins (Tollefsen et al., 2004). This may answer study carried out by Lahnsteiner et al. (2005b). They observed no change of sperm production in *S. trutta fario* exposed to BPA (1.75–5 g L^{-1}) during the spawning period, but sperm concentration significantly decreased in the beginning of the spawning period. These findings suggest that *in vivo* or *in vitro* effects of BPA on sperm characteristics are different and might be varied at different time during the reproductive season. The different effects of BPA might be correspond to dose of exposure, duration of exposure and maturity stage in fish (see reviews by Richter et al., 2007; Gregory et al., 2008; Vandenberg et al., 2009).

In literature, we have found effects of the other EDCs on sperm motility characteristics. In sewage effluents, there are different compounds of Alkylphenols such as 4- Nonylphenol. Lahnsteiner et al. (2005a) did not observe *in vivo* effects of 4-nonylphenol on sperm motility in rainbow trout at concentration up to 750 ng L^{-1}. Recently, Hara et al. (2007) published decrease of motility and velocity of medaka sperm incubated for 60 s in 100μM L^{-1} Nonylphenol. The 2,4-Dichlorophenol (a chlorinated derivative of phenol) also significantly decreased both sperm motility and velocity in African catfish (C*larias gariepinus*) at 1 mg L^{-1}, *in vitro* (Lahnsteiner et al., 2004). In brown trou and burbot (*Lota lota* L.), the 2,4-Dichlorophenol significantly decreased sperm motility at concentrations 0.01 and 10 mg L^{-1}, respectively. But it had no effect on sperm velocity. In contrast, 2,4-Dichlorophenol significantly increased the sperm velocity in *Luciscus cephalus*, but it did not affect percentage of motility of sperm at concentration 100 mg L^{-1}. Reduction of fertility was also reported in medaka exposed to 100 μg L^{-1} 4-nonylphenol (Kang et al., 2002).

In addition, there are some other EDCs such as heavy metals, pesticide and fungicide and paper and mill effluents, which have negative effects on sperm motility. Van Look and Kime (2003) and Kime et al. (1996) observed decrease of sperm motility and velocity in goldfish and African catfish exposed to mercury or zinc, *in vitro*. Among fungicides and pesticides, tributyltin and DDT decreased sperm motility in *Orechromis mossambicus* and *Clarias gariepinus* (Rurangwa et al., 2002; Marchand et al., 2008). McMaster et al. (1992) observed inhibition of sperm motility white sucker (*Catostomus commersoni*) in exposed to paper and mill effluents.

In conclusion, the present study showed that BPA decreases both sperm motility and velocity, *in vitro*. Its mechanisms of action on spermatozoa are not known. They may act through damage of plasma membrane, axonemal apparatus, or depletion of ATP contents in the spermatozoa. The BPA can also reduce the sperm production in fish, which might be through negative impact on production of sex steroids. Alternations of sex steroid production subsequently modify process of sperm maturation that is essential for acquisition of potentiality for sperm motility.

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Author's address: Azadeh Hatef, University of South Bohemia, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, Zatisi 728 ⁄ II, Vodnany 389 25, Czech Republic.
E-mail: hatefa00@vurh.jcu.cz

CHAPTER 3

ENDOCRINE DISRUPTING CHEMICALS ALTER TESTICULAR STEROIDOGENESIS AND SPERM QUALITY, *IN VIVO*

3.1. Hatef, A., Alavi, S.M.H., Abdulfatah, A., Fontaine, P., Rodina, M., Linhart, O., 2012. Adverse effects of Bisphenol A on reproductive physiology in male goldfish at environmentally relevant concentrations. Ecotoxicology and Environmental Safety 76, 56–62.

3.2. Hatef, A., Alavi, S.M.H., Milla, S., Křišťan, J., Fontaine, P., Linhart, O., 2012. Anti-androgen vinclozolin impairs sperm quality and steroidogenesis in goldfish. Aquatic Toxicology 122–123, 181–187.

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Adverse effects of bisphenol A on reproductive physiology in male goldfish at environmentally relevant concentrations

Azadeh Hatef [a,*], Sayyed Mohammad Hadi Alavi [a,*], Abdulbaset Abdulfatah [b], Pascal Fontaine [b], Marek Rodina [a], Otomar Linhart [a]

[a] *South Bohemia Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia, Vodnany 389 25, Czech Republic*
[b] *Unité de Recherche Animal et Fonctionnalités des Produits Animaux, Nancy-Université INRA, 2 avenue de la Forêt de Haye, B.P. 172, 54505 Vandoeuvre-lès-Nancy, France*

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A B S T R A C T

Alternations of reproductive physiology were studied in the male goldfish (*Carassius auratus* L.) exposed to environmentally relevant concentrations (0.6, 4.5 and 11.0 μg/L) of bisphenol A (BPA) at days 10, 20 and 30 after exposure. Significant effects of BPA concentration, exposure time and their interactions were observed on testosterone (T), 11-ketotestosterone (11-KT) and sperm motility and velocity, but gonadosomatic index (GSI), hepatosomatic index (HSI) and 17β-estradiol (E_2) were not affected. Vitellogenin (VTG) was only affected by BPA concentration. The T and 11-KT levels were significantly decreased in the BPA-treated groups after 20 or 30 days. Sperm motility was significantly decreased at 15, 30, 60 and 90 s post-activation in the BPA-treated groups after 20 or 30 days. But, significant decrease in sperm velocity was observed at 30, 60 and 90 s post-activation in the BPA-treated groups at all exposure times. The VTG was significantly increased in the males exposed to 11.0 μg/L at day 30 after exposure. The GSI, HSI and E_2 did not differ between the BPA-treated groups and control. The present study shows that the decrease of sperm quality is concurrent with the decrease of androgens and increase of VTG. The results suggest adverse effects of BPA on sperm motility and velocity via modifications of testicular steroidogenesis that might correspond to alternation in sperm maturation.
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1. Introduction

Bisphenol A (BPA) is widely used for production of polymers (over 2.722×10^9 kg per year) such as polycarbonates, epoxy, phenolic resins, polyesters and polyacrylates (Oehlmann et al., 2008; Vandenberg et al., 2009). These products are used to manufacture polycarbonate plastic products, resins lining cans, dental sealants and food industry. BPA levels were reported 0.02–21 μg/L (Crain et al., 2007) and 0.01–8 μg/L (Kang et al., 2007) in river water. The higher concentrations are also reported near wastewater treatment plants or landfills (Kang et al., 2007).

In mammals, several studies show the adverse effects of BPA on male reproductive physiology and its cellular and molecular modes of action (Crain et al., 2007; Wetherill et al., 2007; Vandenberg et al., 2009). The results show VTG induction at high dose (Gould et al., 1998; Kuiper et al., 1998) and reduction or inhibition of testosterone (T) and 11-ketotestosterone (11-KT) at low dose (Lee et al., 2003; Akingbemi et al., 2004).

In fish, T, 11-KT and 17β-estradiol (E_2) play key roles in spermatogenesis and sperm maturation (Vizziano et al., 2008). The E_2 induces VTG synthesis in the liver, directly (Nelson and Habibi, 2010). The VTG is also known as a biomarker in toxicology, which represents intersex or testicular dysfunction such as alternations in steroidogenesis, sperm production and quality (Denslow and Sepulveda, 2008; Gregory et al., 2008). Data about the effects of BPA on fish reproduction is very rare. Studies show VTG induction in fish exposed to high BPA concentrations (100–1000 μg/L or more) for about 2–4 weeks (Lindholst et al., 2000; Kang et al., 2002; Yamaguchi et al., 2005; Mandich et al., 2007). Decrease of androgens has also been observed in juvenile turbot (Labadie and Budzinski, 2006) and adult common carp (Mandich et al., 2007), but the effective dose was higher than environmentally relevant concentrations. There is also one study that shows adverse effects of BPA on sperm quality in brown trout (*Salmo trutta f. fario*), *in vivo* (Lahnsteiner et al., 2005). This study shows delay in spermiation and decrease in sperm density, motility and velocity in males exposed to BPA at low dose. *In vitro* study on fish sperm showed effective effects on sperm morphology, motility and velocity at very high dose (Hatef et al., 2010). Nevertheless, it is still unclear if alternations in sperm quality are concurrent with gonadal steroidogenesis at environmentally relevant concentration of BPA.

Therefore, the present study was conducted to study the effects of BPA on both steroidogenesis and sperm quality, *in vivo*, in male goldfish (*Carassius auratus* L.) at environmentally

* Corresponding authors. Fax: +420 387774634.
E-mail addresses: ahatef@frov.jcu.cz (A. Hatef), alavi@frov.jcu.cz (S.M.H. Alavi).

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relevant concentrations (0.6, 4.5 and 11.0 μg/L). At days 10, 20 and 30 after exposure, the samples were collected to compare gonadosomatic (GSI) and hepatosomatic indices (HSI), E_2, T, 11-KT, VTG and sperm motility and velocity between the BPA-treated group and those of control.

2. Materials and methods

2.1. Chemicals

The BPA and Dimethyl sulfoxide (DMSO) were purchaced from Sigma-Aldrich (Prague, Czech Republic). The E_2 (ref 582251), T (ref 582701) and 11-KT (ref 582751) EIA kits were purchased from France (SpiBio). Pre-coated ELISA kit for carp VTG was purchased from Biosense laboratories (Bergen, Norway).

2.2. Experimental design

Mature male goldfish (2–3 years-old) were obtained from Hluboka fish farm, Hluboka nad Vltavou, Czech Republic, in April 2009. The health status of fish was firstly checked by random selection of some individuals. Neither parasite nor disease was detected in their gills and mucus. Then, fish were acclimatized for two weeks and distributed into the aquaria (each 80 L) and kept under same temperatures (18–20 °C) and 12 h light /12 h dark photoperiod. Fish were exposed to BPA from May 22, 2009. Water dissolved oxygen and pH were checked three times a week before feeding and were 6.0 ± 0.5 mg/L and 7.5 ± 0.3, respectively. The fish were fed once a day at afternoon with commercial food (ZZN Vodnany, Czech Republic). All animals were handled according to §17 odst. 1 zakona No. 246/119 Sb, Ministry of Agriculture of the Czech Republic, approved by Central Ethics Committee of the Ministry of Health of the Czech Republic.

Experimental groups were composed of three nominal BPA concentrations (1, 6 and 12 μg/L), one solvent control (DMSO) and one control without DMSO. Ten aquaria were used in the present study; two aquaria for each group. Stock of BPA was dissolved in DMSO and then added into each aquarium with final concentration of DMSO at 0.001%. The same volume of DMSO was added to each aquarium. Data of control without DMSO did not show any significant difference with solvent control (data are not shown), therefore only the data for DMSO controls corresponding to each sampling time were used as reference for comparison with respective treatments. Four males were sampled from each aquarium at each sampling time, meaning 8 fish per experimental group. Every 48 h, total volume of water was renewed. There was also no significant difference between groups in terms of fish weight or length (Table 1).

2.3. Evaluation of actual concentrations of BPA

Water samples were collected 1 and 48 h after adding BPA in the aquaria and analyzed using a Triple stage quadrupole MS/MS TSQ Quantum Ultra (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 1250 and Accela 650 LC pumps (Thermo Fisher Scientific, San Jose, CA, USA) and a HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland). Phenyl column (50 mm × 2.1 mm i.d., 5 μm particles) (Thermo Fisher Scientific, San Jose, CA, USA) preceded by a guard column (10 mm × 2.1 mm i.d., 5 μm particles) was used from the same packing material and manufacturer. The highest nominal concentration (20 μg/L) was analyzed by conventional LC injection. A gradient of methanol (MeOH) in water was used for the elution of the BPA and IS. Other samples were analyzed by in-line-SPE-LC–MS/MS, where C18 column (Hypersil Gold, 20 mm × 2.1 mm i.d., 12 μm particles) (Thermo Fisher Scientific, San Jose, CA, USA) was used as an extraction column. Atmospheric pressure chemical ionization interface together with atmospheric pressure photo-ionization in negative ion mode was used for ionization of target compounds. The settings of key parameters were as follows: spray voltage 50 kV, sheath gas 30 and auxiliary gas 15 arbitrary units, vaporizer temperature 300 °C, capillary temperature 325 °C, collision gas was argon at 1.5 mL/min and acetone at flow 50 μL/min was added post-column as a dopant. Both first and third quadrupole were operated at resolution 0.7 FMWH. Two SRM transitions were monitored: 227 → 133 as quantification ion and 227 → 211 as qualification ion. Internal standard method was used for quantification with native Triclosan as internal standard.

Table 1
Body weight and length of male goldfish (*Carassius auratus* L.) exposed to bisphenol A (BPA) at 0.6, 4.5 and 11.0 μg/ L at days 10, 20 and 30 after exposure.

Parameter	BPA	10 day		20 day		30 day	
		n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Weight (g)	Control	6	35.79 ± 4.12	8	34.49 ± 6.01	8	34.10 ± 4.27
	0.6	8	29.92 ± 5.33	8	30.43 ± 4.90	8	35.40 ± 3.20
	4.5	8	30.25 ± 6.70	7	31.50 ± 4.98	8	29.17 ± 3.65
	11.0	8	34.70 ± 6.01	8	33.84 ± 5.53	8	36.21 ± 3.86
Length (cm)	Control	6	13.55 ± 0.46	8	12.58 ± 0.76	8	12.95 ± 0.36
	0.6	8	12.31 ± 0.75	8	12.17 ± 0.79	8	13.09 ± 0.32
	4.5	8	11.84 ± 0.73	7	12.47 ± 0.67	8	12.39 ± 0.67
	11.0	8	13.65 ± 0.98	8	12.76 ± 0.75	8	13.54 ± 0.32

Control group was exposed to 0.001% DMSO. No significant differences were observed between groups at each sampling time ($p > 0.05$).

2.4. Sample collection

Before sampling, each individual was anaesthetized in a 2-phenoxyethanol solution (0.3 mL/L). The sperm was firstly collected by a gentle abdominal massage from the anterior portion of the testis towards the genital papilla and collected with plastic syringes. Attention was taken to avoid sperm contamination by urine, mucus, blood and water. Then, the blood was collected using a heparinized 1 mL syringe from the caudal vein and kept in the eppendorf tubes on ice. Later, each fish was individually weighed (± 0.1 g) and measured for total length (± 1 mm). Fish were then sacrificed and gonads and liver were removed and weighed (± 0.01 g) for determination of GSI (= 100 × gonad weight/total fish weight) and HSI (= 100 × liver weight/total fish weight), respectively. The blood was centrifuged at 5000 rpm for 10 min at 4 °C and plasma was stored in vials at −80 °C until subsequent analyses.

2.5. Sperm motility assessment

To evaluate the sperm quality, sperm of each individual was directly activated in an activation solution (NaCl 50 mM, Tris 20 mM, pH 8.5, 110 mOsmol/kg) at ratio 1:1000–2000 (sperm:activation solution). To avoid sperm sticking to the slides, bovine serum albumin (BSA) was added into the activation medium right before adding sperm at final concentration of 0.1% w/v. Sperm motility was recorded using a 3 CCD video camera (SONY DXC-970MD, Japan) mounted on a dark-field microscope (Olympus BX50, Japan) equipped with stroboscopic lamp. Sensivity of camera is to record 25 frames per second. Then, the successive positions of sperm heads were captured from five frames using a DVD-recorder (SONY DVO-1000 MD, Japan) and analyzed with a micro-image analyzer (Olympus Micro Image 4.0.1. for Windows) (for details see Hatef et al., 2010). Sperm motility (%) and sperm velocity (μm/s) of motile cells were measured from three separate records for each individual and mean of the values was used in statistical analysis.

2.6. Sex steroids measurement

The E_2 was assayed on 50 μL of plasma diluted 1/10 in buffer, using the SpiBio EIA kit. Sensitivity was 47 pg/mL, and intra-assay and inter-assay coefficients of variation were, respectively, 8% and 17% for a plasma concentration of 2280 pg/mL. The T required a preliminary extraction in diethyl ether (Fontaine et al., 2006). Fifty microliters of plasma diluted to one-tenth in ether was frozen under −20 °C after agitation. The supernatant containing the sex steroids was collected and the contents evaporated under vacuum bell-jar. The sample was then included in the same volume of buffer and assayed in using the SpiBio EIA kit. Sensitivity was 40 pg/mL and intra-assay and inter-assay coefficients of variation were, respectively, 4.7% and 10.6% for a plasma concentration of 1610 pg/mL. The 11-KT was assayed on 50 μL of plasma diluted 1/20 in buffer, using the SpiBio EIA kit. Sensitivity was 190 pg/mL and intra-assay and inter-assay coefficients of variation were 4.1% and 5.2% for a plasma concentration of 2240 pg/mL, respectively.

2.7. VTG assessment

Measurement of VTG in the blood plasma was performed using a pre-coated ELISA kit (Biosense laboratories® Norway). The use of the ELISA kit for determination of VTG has been already validated in cyprinids (Žlábek et al., 2009). Absorbance was measured using an SLT Spectra (A5082) instrument at 492 nm for VTG. The regression obtained for absorbance was $y = 0.06 \times ^{0.97}$, $r^2 = 0.99$.

2.8. Statistical analysis

All data were analyzed using SPSS 9.0 for windows. Homogeneity of variance was tested for all data using Levene's test. As needed, data were transformed to meet assumptions of normality, and homoscedasticity. Data of T and VTG levels were $\log_{10}$-transformed. Repeated measures analysis of variance (ANOVA) was used to understand the effects of BPA concentration, exposure time and their interactions on each measured parameters (for details see Hatef et al., 2011); alpha was set at 0.05. When significant BPA concentration × exposure time interactions was detected, the model was revised into individual ANOVA models at each exposure time and analyzed using a single-factor ANOVA followed by

Tukey's post-hoc test. When no significant BPA concentration × exposure time interaction was observed, the model was re-run with the interaction effect removed, and main effects (BPA concentration and exposure time) were then interpreted. All data are presented as mean ± standard error of mean (S.E.M.).

3. Results

One hour after adding BPA into the aquaria, the actual BPA concentrations (mean ± SD) were undetectable in the solvent control and were measured 0.61 ± 0.03, 4.50 ± 0.70, 11.01 ± 0.55 μg/L for 1, 6 and 12 μg/L BPA, respectively. After 48 h, the actual BPA concentration decreased to 0.25 ± 0.17, 0.72 ± 0.59 and 2.26 ± 0.64 μg/L for 1, 6 and 12 μg/L, respectively.

From repeated measures ANOVAs, significant effects of BPA concentration, exposure time and their interactions were observed on T, 11-KT and sperm motility and velocity, but GSI, HSI and E_2 were unaffected (Table 2). The VTG was only affected by BPA concentration (Table 2). Therefore, the models for GSI, HSI, E_2 and VTG were re-run with the BPA concentration × exposure time interactions' effects removed and the effects of BPA concentration and exposure times were interpreted. Neither BPA concentration nor exposure time influenced GSI ($F_{\text{BPA concentration}}=0.88_3$, $F_{\text{exposure time}}=1.77_2$), HSI ($F_{\text{BPA concentration}}=1.15_3$, $F_{\text{exposure time}}=3.05_2$) and E_2 ($F_{\text{BPA concentration}}=2.04_3$, $F_{\text{exposure time}}=2.83_2$) ($p>0.05$). Table 3 shows GSI, HSI and E2 in the BPA-treated groups and control at different exposure time. The VTG was significantly affected by BPA concentration ($F=6.59_3$, $p<0.01$), but exposure time did not influence VTG ($F=1.10_2$, $p>0.05$). Separate ANOVA for each exposure time showed significant effect of BPA concentrations on VTG only at 30 day exposure (Table 4). In this time, significant increase of VTG was observed in the males exposed to 11.0 μg/L (Fig. 1).

For T, 11-KT and sperm motility and velocity, the models were revised into three separate repeated ANOVAs for each exposure time. The T and 11-KT were not affected by BPA concentrations at day 10, but were significantly affected after 20 and 30 days (Table 4). Compared to the control, T was significantly decreased at 4.5 and 11.0 μg/L BPA after day 20 and at 11.0 μg/L BPA after day 30 (Fig. 2). At day 20, 11-KT was decreased at 4.5 and 11.0 μg/L BPA compared to that of control (Fig. 3). Significant decrease of 11-KT was also observed at 0.6 and 4.5 μg/L BPA after day 30 (Fig. 3).

Significant effects of BPA concentrations on sperm motility were observed at day 20 and 30 (Table 4, Fig. 4). At day 10 after exposure, sperm motility did not differ between the BPA-treated groups and control at any time post-activation (Table 4, Fig. 4). Sperm motility, compared at 15 s post-activation, was significantly lower in males exposed to BPA at 4.5 and 11.0 μg/L for day 20 or 30 than the control

Table 3
Gonadosomatic index (GSI, %), hepatosomatic index (HIS, %) and estradiol (E_2, pg/mL) in male goldfish (*Carassius auratus* L.) exposed to bisphenol A (BPA) at 0.6, 4.5 and 11.0 μg/L at days 10, 20 and 30 after exposure.

Parameter	Group	10 day	20 day	30 day
GSI	Control	2.96 ± 0.64	2.68 ± 0.43	2.66 ± 0.35
	0.6	3.07 ± 0.54	2.21 ± 0.38	3.35 ± 0.54
	4.5	3.77 ± 0.69	3.01 ± 0.44	2.76 ± 0.43
	11.0	2.75 ± 0.50	2.20 ± 0.46	2.72 ± 0.43
HSI	Control	1.89 ± 0.22	1.37 ± 0.19	1.53 ± 0.12
	0.6	1.90 ± 0.21	1.82 ± 0.47	1.75 ± 0.21
	4.5	2.18 ± 0.13	1.87 ± 0.19	1.61 ± 0.09
	11.0	2.02 ± 0.22	1.83 ± 0.16	1.59 ± 0.22
E_2	Control	253.90 ± 70.14	192.45 ± 20.69	190.25 ± 11.12
	0.6	200.17 ± 17.65	173.50 ± 13.72	168.38 ± 31.74
	4.5	187.75 ± 20.89	186.43 ± 13.73	151.20 ± 20.65
	11.0	192.10 ± 12.32	133.55 ± 23.24	154.75 ± 21.20

Control group was exposed to 0.001% DMSO. No significant differences were observed between groups ($p>0.05$).

Table 4
Summary of statistics (F_{df}) obtained from separate ANOVA models used to study the effects of bisphenol A (BPA) concentration on testosterone (T), 11-ketotestosterone (11-KT), vitellogenin (VTG) and sperm motility and velocity in goldfish (*Carassius auratus* L.) at different exposure times.

Parameter	Times post-sperm activation (s)	Exposure time		
		10	20	30
T		0.33_3	6.49_3^{**}	2.80_3^{*}
11-KT		0.49_3	7.89_3^{***}	3.99_3^{*}
VTG		1.27_3	1.75_3	3.69_3^{*}
Sperm motility	15	1.39_3	17.58_3^{***}	3.05_3^{*}
	30	0.60_3	7.54_3^{***}	4.63_3^{**}
	60	1.46_3	5.99_3^{***}	9.64_3^{***}
	90	2.23_3	6.90_3^{***}	13.83_3^{***}
Sperm velocity	15	0.77_3	1.12_3	1.30_3
	30	3.49_3^{*}	3.34_3^{*}	2.71_3^{*}
	60	3.01_3^{*}	4.53_3^{**}	2.18_3^{*}
	90	4.05_3^{*}	10.94_3^{***}	4.56_3^{**}

Table 2
Summary of statistics (F_{df}) obtained from repeated measures ANOVA models used to study the effects of bisphenol A (BPA) concentration, exposure time and their interaction on gonadosomatic (GSI) and hepatosomatic (HSI) indices, estradiol (E_2), testosterone (T), 11-ketotestosterone (11-KT), vitellogenin (VTG) and sperm motility and velocity in goldfish (*Carassius auratus* L.).

Parameter	Times post-sperm activation (s)	BPA concentration	Exposure time	BPA concentration × exposure time
GSI		0.83_3	1.55_2	0.54_6
HSI		0.93_3	3.03_2	0.36_6
E_2		2.22_3	3.00_2	0.37_6
T		4.603^{**}	19.91_2^{***}	2.71_6^{*}
11-KT		2.94_3^{*}	10.40_2^{***}	4.23_6^{**}
VTG		5.31_3^{**}	1.34_2	0.64_6
Sperm motility	15	79.4_3^{***}	85.6_2^{***}	52.3_6^{***}
	30	42.9_3^{***}	60.9_2^{***}	28.7_6^{***}
	60	15.8_3^{***}	23.8_2^{***}	7.1_6^{***}
	90	23.8_3^{***}	32.8_2^{***}	6.9_6^{***}
Sperm velocity	15	1.6_3	5.2_2^{**}	3.4_6^{*}
	30	5.1_3^{**}	21.2_2^{***}	2.8_6^{**}
	60	4.0_3^{**}	1.6_2	3.7_6^{**}
	90	2.7_3^{**}	0.4_2	5.2_6^{***}

* $p<0.05$.
** $p<0.01$.
*** $p<0.001$.

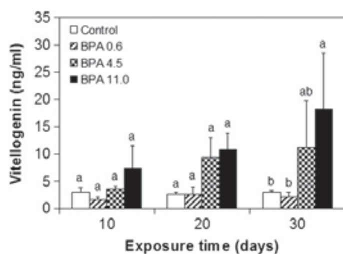

Fig. 1. Vitellogenin in the blood plasma of male goldfish (*Carassius auratus* L.) exposed to bisphenol A at different exposure times. Values with different superscripts are significantly different ($p < 0.05$). Control group was exposed to 0.001% DMSO.

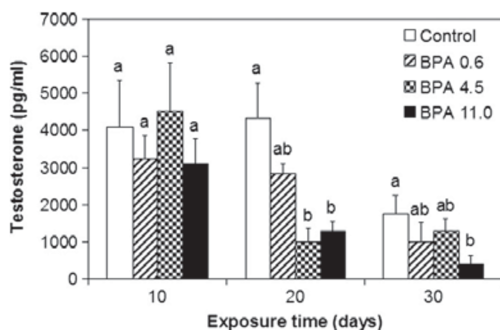

Fig. 2. Testosterone in the blood plasma of male goldfish (*Carassius auratus* L.) exposed to bisphenol A at different exposure times. Values with different superscripts are significantly different ($p < 0.05$). Control group was exposed to 0.001% DMSO.

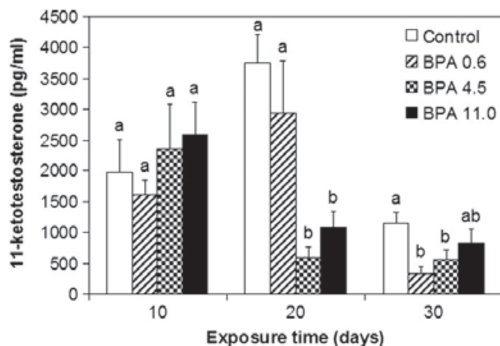

Fig. 3. 11-Ketotestosterone in the blood plasma of male goldfish (*Carassius auratus* L.) exposed to bisphenol A at different exposure times. Values with different superscripts are significantly different ($p < 0.05$). Control group was exposed to 0.001% DMSO.

group (Fig. 4a). When sperm motility was compared at 30 s post-activation, significant decrease was observed in all BPA-treated groups after day 20 and at 4.5 and 11.0 μg/L BPA after day 30 (Fig. 4b). At 60 s post-activation, sperm motility was lower at 4.5 and 11.0 μg/L BPA after day 20 and in all BPA-treated groups after day 30 (Fig. 4c). At 90 s post-activation, sperm motility showed significant decrease at 4.5 μg/L BPA after day 20 and in all BPA-treated groups after day 30 (Fig. 4d).

Sperm velocity at 15 s post-activation did not differ between the BPA-treated groups and control at any exposure time (Table 4, Fig. 5). Sperm velocity at 30, 60 and 90 s post-activation showed significant differences between the BPA-treated groups and control at days 10, 20 and 30 after exposure (Table 4, Fig. 5). Decrease of sperm velocity at 30 s post-activation was observed at 4.5, 0.6 and 11.0 μg/L BPA after days 10, 20 and 30, respectively (Fig. 5b). When the sperm velocity was compared at 60 s post-activation, significant decrease was observed in all BPA-treated groups after day 10 and at 4.5 μg/L BPA after days 20 and 30 (Fig. 5c). At 90 s post-activation, sperm velocity was lower in all BPA-treated groups (Fig. 5d).

4. Discussion

The present study showed that disruption of male reproductive physiology in goldfish exposed to environmentally relevant concentrations of BPA via alternations of androgens (T and 11-KT) and VTG synthesis and sperm motility and velocity.

It is known that microbial degradation and BPA adsorption to organic matter in the water, fish or tanks can give rise to deviations from a nominal concentration (Staples et al., 1998; Lindholst et al., 2000). Therefore, the actual concentrations of BPA were measured in the present study at time of adding (1 h) and renewing of water (48 h). Decreasing BPA concentration from 1 to 48 h suggests its bioaccumulation in the tissues such as liver, gonad and muscle, which has been previously reported in fish or other aquatic organisms (Staples et al., 1998; Lindholst et al., 2000; Li et al., 2009). Moreover, in the present study, no mortality was observed in the BPA-treated groups, indicating that the examined doses of BPA were not toxic.

To our knowledge, decrease of androgens observed in the present study at environmentally relevant concentrations of BPA is novel in fish and needs to be investigated in detail. Probably, BPA acts as anti-androgen at low dose similar to mammals (Lee et al., 2003; Akingbemi et al., 2004), however the mechanism involved should be studied with emphasis on neurosteroids and transcriptomics response of steroid receptors to BPA in reproductive organs. These studies are currently underway in our laboratory. The results of sex steroids are in agreement with Folmar et al. (1996) that observed lower T in male common carp (*Cyprinus carpio*) sampled below sewage treatment plants containing high BPA concentrations. In fish, decrease of T has been previously reported, but the effective dose of BPA was extremely high, for example in mature male common carp exposed to 1000 μg/L BPA for 14 days (Mandich et al., 2007), in juvenile turbot (*Psetta maxima*) exposed to 59 μg/L BPA for 21 days (Labadie and Budzinski, 2006) and in larvae brown trout (*Salmo trutta*) exposed to 50 μg/L BPA for 63 days (Bjerregaard et al., 2008). It is unknwon why the various effective doses of BPA on androgen synthesis have been observed in different studies; it might be addressed to gonadal developmental stage, experimental design and/or species-specific response to BPA.

Data about effects of BPA on sperm quality in fish are very limited. However, observed decrease of sperm motility and velocity in the present study is in agreement with the work of Lahnsteiner et al. (2005) that showed lower sperm motility and velocity in the brown trout exposed to 1.75 or 2.40 μg/L BPA, *in vivo*. They hypothesized that BPA causes modulations in sperm maturation, but their study lacked data about sex steroids. To our knowledge, this is the first study that shows decrease of sperm quality concurrent with the decrease of androgens. This suggests that the adverse effects of BPA on sperm motility and velocity via

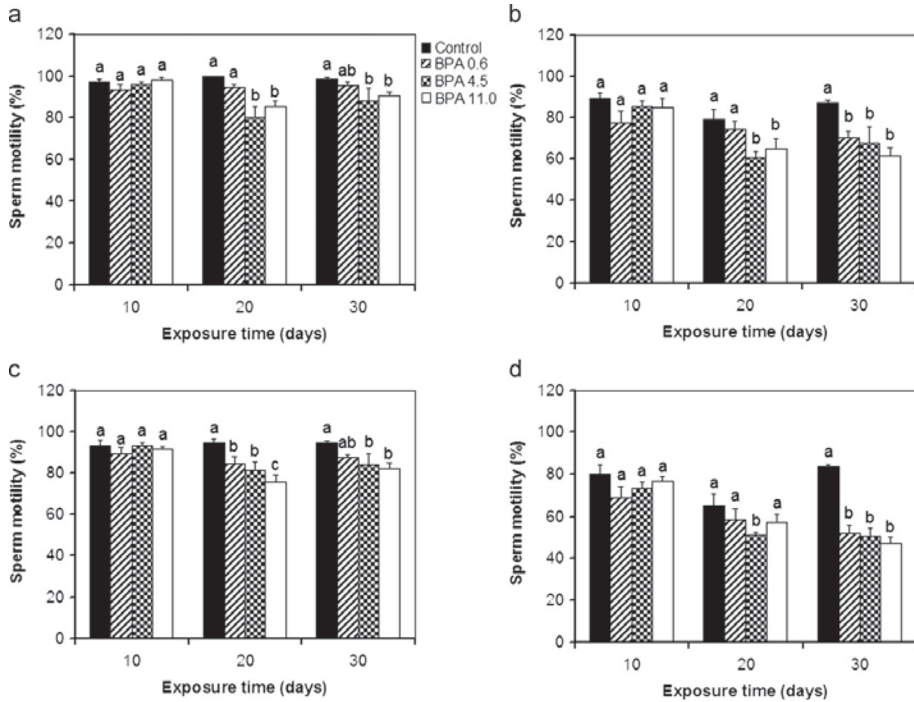

Fig. 4. Sperm motility (%) in goldfish (*Carassius auratus* L.) exposed to different concentrations of bisphenol A (BPA) at 15 (a), 30 (b), 60 (c) and 90 (d) s post-activation. The males were exposed to BPA at 0.6, 4.5 and 11.0 μg/L for 10, 20 and 30 days. Control group was exposed to 0.001% DMSO. Values with the same letters are not significantly different at each exposure time ($p > 0.05$).

alternations of the final sperm maturation in the testicular main and spermatic ducts (Alavi et al., 2008; Vizziano et al., 2008). The adverse effect of BPA on sperm motility via modulation of sex steroidogenesis becomes interesting when results of the present study are compared with our previous work (Hatef et al., 2010). In our previous work, we have observed that sperm motility and velocity decrease when 228×10^3 μg/L (1 mM) was added into the activation medium, *in vitro* (Hatef et al., 2010). This shows that extremely high and very low doses of BPA are required to decrease the sperm motility and velocity, *in vitro* and *in vivo*, respectively. In mammals, a dose-dependent reduction in epididymal sperm motility has been reported following oral administration of BPA to male rats at doses of 0.2–20 μg BPA per kg body weight per day for a period of 45 days (Chitra et al., 2003). Meeker et al. (2010) recently reported relationship between sperm motility and urinary BPA in men from an infertility clinic. Though evidences demonstrate adverse effects of BPA on sperm motility, its modes of action are still unknown. Maturation of sperm in fish is regulated by intracellular signaling such as increase of cAMP and pH (Miura et al., 1992; Cosson et al., 1999). Sperm motility and velocity are also depending on initial ATP content, plasma membrane potential for triggering the flagellar beating and Ca^{2+} signaling (Alavi et al., 2008; Cosson, 2010). Therefore, future directions can be addressed to physiological and biochemical alternations of sperm in fish exposed to BPA. For example, *in vitro* study on the effects of $HgCl_2$ on fish sperm showed its effects through the damage of sperm cells (plasma membrane, mitochondria and axoneme) and ATP content of sperm (Hatef et al., 2011). In addition to these parameters, cytoskeletal proteins in sperm and DNA damage could be considered as indicators for qualification of sperm (Peknicova et al., 2002; Meeker et al., 2010).

Although BPA influences hormonal functions of gonad and sperm motility, but no effects on the weight of reproductive organ have been reported in the present or previous studies (Kang et al., 2002; Peknicova et al., 2002; Ishibashi et al., 2005; Mandich et al., 2007). Mandich et al. (2007) did not show significant changes in GSI and HSI of adult common carp exposed to 1–1000 μg/L BPA for 15 days. Higher concentrations of BPA (3120 μg/L) did not also change GSI and HSI in male medaka exposed for 21 days (Kang et al., 2002; Ishibashi et al., 2005). In literature, Hassanin et al. (2002) and Patino et al. (2003) observed the adverse effects of endocrine disrupting chemicals on GSI in common carp captured from highly polluted rivers that might be due to combination of BPA with other EDCs, mostly estrogenic chemicals such as E_2, nonylphenol or octylphenol.

Decrease of E_2 has been shown in fish or mammals exposed to BPA at environmentally relevant concentrations (Akingbemi et al., 2004; Mandich et al., 2007). But, in the present study, similar to Bjerregaard et al. (2008), no significant change in E_2 was observed between control and BPA-treated fish. The differences among studies might be addressed to initial levels of androgens and E_2 in the experimental animals and their sensitivity for responding to BPA (Denslow and Sepulveda, 2008; Gregory et al., 2008). Although, E_2 unchanged in the present study, but VTG production was significantly affected. This also suggest modulation of E_2-mediated VTG

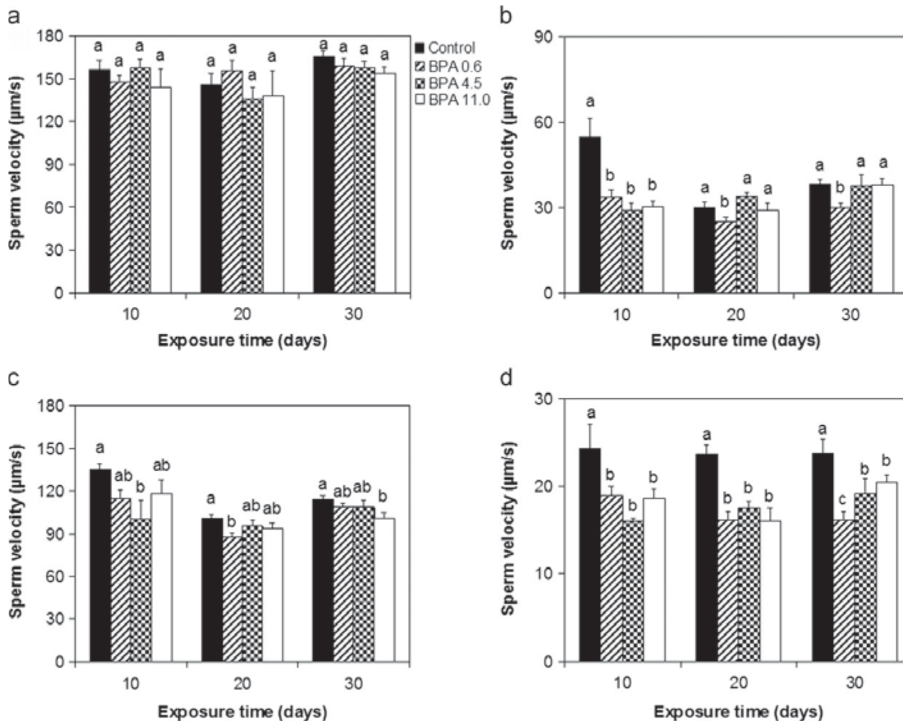

Fig. 5. Sperm velocity (μm/s) in goldfish (*Carassius auratus* L.) exposed to different concentrations of bisphenol A (BPA) at 15 (a), 30 (b), 60 (c) and 90 (d) s post-activation. The males were exposed to BPA at 0.6, 4.5 and 11.0 μg/L for 10, 20 and 30 days. Control group was exposed to 0.001% DMSO. Values with the same letters are not significantly different at each exposure time ($p > 0.05$). Velocity of motile spermatozoa was used in analyses.

synthesis in liver of fish exposed to BPA, which needs to be investigated in further studies. Induction of VTG at 11.0 μg/L BPA observed in the present study also suggests estrogenic activity of BPA at high environmentally relevant concentrations. Different studies show VTG induction in a dose- and time-dependent manner. For example, significant increase in VTG has been reported in adult common carp (Mandich et al., 2007) and medaka (Kang et al., 2002; Ishibashi et al., 2005) at 1000 μg/L BPA after 14 and 21 days exposure, respectively. Lindholst et al. (2000) observed VTG induction in rainbow trout exposed to 500 μg/L BPA for 12 days through a continuos flow system. In Atlantic cod, BPA at 59 μg/L induced VTG production after 21 days exposure (Larsen et al., 2006). In fathead minnow, significant induction in VTG production was observed 43 or 71 days after exposure at 460 or 160 μg/L BPA (Sohoni et al., 2001). The differences among studies could be addressed to BPA uptake rates, species-specific ER binding affinities, fish species, age, maturity stage and exposure period (Tyler et al., 1996; Lindholst et al., 2000; Crain et al., 2007).

5. Conclusions

The BPA at environmentally relevant concentrations could produce potential harm to fish reproduction through alternations of sex steroidogenesis and VTG induction. The present study showed decrease of androgens, which involve in spermatogenesis and sperm maturation in males exposed to BPA at environmentally relevant concentration. Alternations of androgen synthesis lead to decrease of sperm motility and velocity, probably via disruption of sperm maturation. Also, BPA at high environmentally relevant concentration can induce VTG in fish and exhibit estrogenic mode of action. Therefore, further studies are required to study mechanisms in which BPA influence sperm maturation. Decrease of androgens and increase of VTG are evidences for anti-androgenic and estrogenic modes of action of BPA, respectively, that are required to be investigated at molecular level.

Conflict of interest statement

There is no conflict of interests.

Acknowledgments

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Anti-androgen vinclozolin impairs sperm quality and steroidogenesis in goldfish

Azadeh Hatef[a], Sayyed Mohammad Hadi Alavi[a,*], Sylvain Milla[b], Jiří Křišťan[a], Mahdi Golshan[a], Pascal Fontaine[b], Otomar Linhart[a]

[a] University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodňany 389 25, Czech Republic

[b] Université de Lorraine, UR. AFPA, Equipe DAC, 2 Avenue de la Forêt de Haye, Vandoeuvre-les-Nancy F-54505, BP 172, France

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ABSTRACT

In mammals, vinclozolin (VZ) is known as anti-androgen, which causes male infertility via androgen receptor (AR) antagonism. In aquatic animals, the VZ effects on reproductive functions are largely unknown and results are somewhat contradictory. To understand VZ adverse effects on male reproduction, mature goldfish (*Carassius auratus*) were exposed to three nominal VZ concentrations (100, 400, and 800 μg/L) and alternations in gonadosomatic (GSI) and hepatosomatic indices (HSI), 17β-estradiol (E_2), 11-ketotestosterone (11-KT) and sperm quality were investigated compared to the solvent control. One group was exposed to E_2 (nominal concentration of 5 μg/L), an estrogenic compound, as a negative control. Following one month exposure, GSI and HSI were unchanged in all VZ treated groups compared to solvent control. Sperm volume, motility and velocity were reduced in fish exposed to 800 μg/L VZ. This was associated with the decrease in 11-KT level, suggesting direct VZ effects on testicular androgenesis and sperm functions. In goldfish exposed to 100 μg/L VZ, 11-KT was increased but E_2 remained unchanged. This is, probably, the main reason for unchanged sperm quality at 100 μg/L VZ. In goldfish exposed to E_2, GSI and 11-KT were decreased, E_2 was increased and no sperm was produced. The present study shows different dose-dependent VZ effects, which lead to impairment in sperm quality via disruption in steroidogenesis. In addition to VZ effects through competitive binding to AR, our data suggests potential effects of VZ by direct inhibition of 11-KT biosynthesis in fish as well as abnormalities in sperm morphology.

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1. Introduction

Most studies on reproductive dysfunctions in aquatic species have focused on endocrine disrupting chemicals (EDCs) exhibiting estrogenic action (Leet et al., 2011). Comparatively little attention has been given to chemicals that might interact with androgens, due to the fact that the functions of androgen receptors (AR) are largely unknown (Borg, 1994). It is clear that androgens (testosterone, T and 11-ketotestosterone, 11-KT) are essential to control sexual differentiation and spermatogenesis in males by binding to AR to activate or repress the expression of specific genes (Borg, 1994; Delvin and Nagahama, 2002; Leet et al., 2011). In this context, fish and mammals are different. The 11-KT is a key androgen, more effective than T, which stimulates spermatogonial proliferation and subsequent spermatogenesis stage as well as secondary sexual characters in fish (Borg, 1994; Young et al., 2005). But in mammals, T controls spermatogenesis and sexual characteristics (McLachlan et al., 2002; Sofikitis et al., 2008). Androgen functions could be adversely affected by anti-androgens that mimic or block the androgenic responses via AR antagonist mode of action (Kelce et al., 1997). Several classes of pesticides are demonstrated as anti-androgens, which interfere with testicular functions and cause severe impairment in male fertility via inhibition of androgen biosynthesis (MacLatchy et al., 1997; Sharpe et al., 2004; Hatef et al., 2012).

Vinclozolin is a dicarboximide fungicide widely used in Europe and the United States for control of diseases caused by *Botrytis cineera*, *Sclerotinia sclerotiorum* and *Monilinia* species in grapes, fruits, vegetables, ornamental plants, and turfgrass (Pothuluri et al., 2000). It had not been considered to be hazardous to vertebrates (U.S. EPA, 1998). But further studies in mammals indicated that VZ might exert anti-androgenic activity, and the effects may persist over the next generations. These effects include disruption in testicular development and steroidogenesis as well as decrease in sperm quality (Kubota et al., 2003; Kang et al., 2004; Anway et al., 2005; Elzeinova et al., 2008; Eustache et al., 2009). These studies suggest that the adverse effects of VZ are mediated by alternations

* Corresponding author at: University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, Vodňany 389 25, Czech Republic. Tel.: +420 387 774 610; fax: +420 387 774 634.
E-mail address: alavi@frov.jcu.cz (S.M.H. Alavi).

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in neuroendocrine regulation of reproduction which increase T biosynthesis, but cause male infertility via inhibiting binding of T to the AR (Kelce et al., 1997; Loutchanwoot et al., 2008).

Compared to mammals, studies on VZ effects are very rare in fish and have been focused on gonadal differentiation, testicular development and endocrine control of reproduction (Table 1). These studies show various effects of VZ on male reproduction including occurrence of intersex (Koger et al., 1999), disruption of gonadal development (Kiparissis et al., 2003), decrease in sexual characteristics, sperm counts, and performance of sexual behavior (Baatrup and Junge, 2001; Bayley et al., 2002, 2003) and disruption in testicular steroidogenesis (Martinovic et al., 2008; Villeneuve et al., 2007). In contrast, Makynen et al. (2000) reported no alteration in gonadal differentiation and T, 11-KT or 17β-estradiol (E_2) biosyntheses as well as no VZ affinity to AR. Taken together, the literature on anti-androgenic effects of VZ in fish is somewhat contradictory and there is a lack of information on its potential effects on sperm quality as a critical endpoint for male fertility.

Therefore, we evaluated the effects of VZ on goldfish (*Carassius auratus*) reproduction when the broodfish were exposed to 100, 400 and 800 μg/L VZ for one month. A group of fish was exposed to E_2 as a negative control for better understanding the modes of action of VZ. The main objective was to study sperm quality (volume, motility and velocity) as endpoints for male fertility. Additionally, the ability of VZ to modulate sex steroid production (11-KT and E_2) was investigated. The present study is a comprehensive assessment of VZ toxicity in male goldfish, showing a decrease in sperm quality associated with disruption in steroidogenesis in fish exposed to VZ. Previous studies on fish showed similarity to mammals in which VZ acts through AR while 11-KT level increases (Table 1). But the present study suggests potential VZ effect via inhibition of 11-KT biosynthesis.

2. Materials and methods

2.1. In vivo exposure of adult goldfish

Mature male goldfish (2–3 years old) were used after checking their health status. Fish were first acclimatized for two weeks and then distributed into the aquaria (each 80 L) and kept under same temperatures (18–22 °C, at the beginning and end of experiment, respectively) and 12 h light/12 h dark photoperiod. Dissolved oxygen level and pH of water were checked three times a week before feeding and were 6.0 ± 0.5 mg/L and 7.5 ± 0.3, respectively. The fish were fed once a day (3% body weight) with commercial food (ZZN Vodnany, Czech Republic). All animals were handled according to §17 odst. 1 zakona No. 246/119 Sb, Ministry of Agriculture of the Czech Republic, approved by Central Ethics Committee of the Ministry of Health of the Czech Republic.

Experimental groups were composed of fish exposed to three nominal VZ concentrations (100, 400, and 800 μg/L), one solvent control (acetone), and one negative control (E_2, 5 μg/L). Ten aquaria were used in the present study; two aquaria for each group. Stock of VZ was dissolved in acetone and then added into each aquarium with final concentration of acetone at 0.001%. The same volume of acetone was added to every aquarium. Three males were sampled from each aquarium (n = 6 per each experimental group). Every 48 h, total volume of water was renewed.

Before sampling, each individual was anaesthetized in a 2-phenoxyethanol solution (0.3 ml/L). The sperm was first collected by a gentle abdominal massage from the anterior portion of the testis towards the genital papilla and collected with plastic syringes. Attention was taken to avoid sperm contamination by urine, mucus, blood or water. Then, the blood was collected using a heparinized 1 ml syringe from the caudal vein and kept in the eppendorf tubes on ice. Each fish was individually weighed (±0.1 g) and measured for total length (±1 mm). Fish were then sacrificed and gonads and liver were removed and weighed (±0.01 g) for determination of GSI (=100 × gonad weight/total fish weight) and HSI (=100 × liver weight/total fish weight). The blood was centrifuged at 5000 rpm for 10 min at 4 °C and plasma was stored in 1.5 mL eppendorf tubes at −80 °C until sex steroid analyses.

2.2. Sperm quality

Sperm volume was measured for each individual and expressed as μL. To evaluate the sperm motility, sperm of each individual was directly activated in an activation solution (NaCl 50 mM, Tris 20 mM, pH 8.5, 110 mOsmol/kg) at ratio 1:1000–2000 (sperm:activation solution). To avoid sticking of sperm into the slides, bovine serum albumin (BSA) was added into the activation medium right before adding sperm at final concentration of 0.1% (w/v). Sperm motility was recorded for frames captured by a CCD video camera (SONY DXC-970MD, Japan) mounted on a dark-field microscope (Olympus BX50, Japan) using a DVD-recorder (SONY DVO-1000 MD, Japan). A micro image analyzer (Olympus Micro Image 4.0.1. for Windows) was used to analyse sperm motility (% of total sperm) and velocity of only motile spermatozoa (μm/s) based on the successive positions of sperm heads (for details see Hatef et al., 2010). Two separate records for each individual and the mean of these values were used in statistical analysis.

2.3. Sex steroid measurements

The E_2 was assayed on 50 μL of plasma diluted from 1/3 to 1/10 in a specific buffer, using the Diasource EIA kit (Diasource, Nivelles, Belgium). 50 μL of each E_2 standard, control and plasma samples were dispensed in each well of a 96-well plate. 50 μL of E_2-horseradish peroxidase conjugate were added into each well as well as 50 μL of antibody directed against E_2. After 120 min incubation at room temperature, and 4 times washing, 200 μL of substrate solution were added. Then, the plate was incubated for 30 min at room temperature and the reaction was stopped by adding 100 μL of H_2SO_4 (1.8 N). The optical density was read at 450 nm with a microtiter plate reader. Sensitivity was 5 pg/mL, and intra-assay and inter-assay coefficients of variation were, respectively, 4% and 6% for a plasma concentration in the range of 100–250 pg/mL. Concerning 11-KT, the Cayman EIA kit was used (Cayman, Michigan, USA) with plasma diluted 10 times. Similar to E_2 assay, 50 μL of plasma/standards, tracer and antibody were added into each well. After 120 min incubation and 5 times washing, 200 μL of substrate solution were added. This step was followed by a 90 min incubation step before reading the absorbance at 405 nm. Sensitivity was 1.3 pg/mL and intra-assay and inter-assay coefficients of variation were, respectively, 8% and 9% for a plasma concentration of 6 pg/mL. Sex steroids were measured twice for each sample and mean of these values were used in statistical analysis.

2.4. Statistical analysis

Homogeneity of variance was tested for all data using Levene's test. Data for 11-KT and E_2 were log transformed to meet assumptions of normality and homoscedasticity. Tukey–Kramer test was used in conjunction with an ANOVA to find which means are significantly different from one another. In this context data of VZ-treated groups were compared with solvent control and alpha was set at 0.05. Similar statistical analyses were performed to compare negative control (E_2) with VZ-treated groups and solvent control. All data are presented as mean ± standard error of mean (S.E.M.).

Table 1
Summary of studies performed to investigate the effects of vinclozolin on reproductive physiology in fish.

Fish species	Concentration (μg/L)	Exposure period	Developmental stage	Endpoints	Authors
Goldfish	100, 400, 800	30 days	Maturity stage	No change in GSI Increase and decrease in 11-KT at 100 and 800, respectively No change in E_2 Decrease in sperm volume, motility and velocity at 800	Present study
Zebrafish	10	14, 48 and 72 h	Embryonic stage	Increase in AR mRNA	Smolinsky et al., 2010
Fathead minnow	100, 400 and 700	21 days	Maturity stage	No mortality Increase in GSI at 400 and 700 Decrease in reproductive frequency at 700 Decrease in male-specific sexual characters at 400 and 700 No alternations in testicular development Increase in 11-KT at 400 and 700, ex vivo No significant change in T and E_2 Increase in testicular AR at 700	Martinovic et al., 2008
Fathead minnow	100, 400 and 700	21 days	Maturity stage	Increase in pituitary FSHβ mRNA at 700, but LHβ remain unchanged Increase in testicular LHR at 100, 400 and 700, but no change in FSHR	Villeneuve et al., 2007
Medaka	2500	100 days	From 1 to 100 day post hatch	Spermatogenesis inhibition No intersex Decrease in sperm density evaluated by histological sections of testis	Kiparissis et al., 2003
Guppy[a]	0.1, 1 and 10	30 days	Maturity stage	No mortality Decrease in sperm count at 1 and 10	Bayley et al., 2003
Guppy[a]	0.1 and 10		Juvenile development	No mortality Delay in sexual maturation Decrease in sperm count at 1 and 10	Bayley et al., 2002
Guppy[a]	1, 10 and 100	21 days	Maturity stage	15% mortality at 1 No effects on testicular development	Kinnberg and Toft, 2003
Guppy[a]	1 and 10	30 days	Maturity stage	Decrease in GSI No change in sperm count Decrease in sexual behavior	Baatrup and Junge, 2001
Fathead minnow	75–1200	34 days	Embryonic stage	No effect on survival rate No change in sex ratio No change in fecundity	Makynen et al., 2000
Fathead minnow	200 and 700	21 days	Maturity stage	No mortality No change in GSI No alternations in testicular development No change in T and 11-KT Increase in E_2 at 700 No affinity of VZ binding to T binding sites in brain up to 50 μM	Makynen et al., 2000

11-KT: 11-ketotestosterone; AR: androgen receptor; E_2: 17β-estradiol; FSH: follicle stimulating hormone; FSHR: follicle stimulating hormone receptor; GSI: gonadosomatic index; LH: luteinizing hormone; LHR: luteinizing hormone receptor; T: testosterone.
[a] Vinclozlin has been administrated by food (μg/mg food).
Note: For transcriptomic alternations in reproductive genes, see Villeneuve et al. (2007) and Martinovic et al. (2008); data are not included.

Table 2
Total length (cm), body mass (g), gonadosomatic index (GSI, %) and hepatosomatic index (HSI, %) of male goldfish exposed to vinclozolin (VZ; 100, 400, and 800 μg/L) and 17β-estradiol (5 μg/L) for one month. Data are mean ± S.E.M. ($n = 6$). For each parameter, values with different superscripts are significantly different ($p < 0.05$).

VZ (μg/L)	Total length	Body mass	GSI	HSI
Control	11.5 ± 0.3^a	25.8 ± 2.2^a	2.1 ± 0.7^a	2.6 ± 0.2^a
100	13.6 ± 0.2^a	32.0 ± 2.3^a	2.9 ± 0.5^a	2.9 ± 0.2^a
400	12.6 ± 0.4^a	32.6 ± 2.3^a	1.9 ± 0.3^a	2.9 ± 0.3^a
800	13.0 ± 0.2^a	28.1 ± 4.1^a	1.7 ± 0.4^a	2.9 ± 0.4^a
17β-Estradiol	12.0 ± 0.5^a	24.6 ± 2.4^a	0.9 ± 0.3^b	2.9 ± 0.3^a

3. Results

3.1. Fish body mass, total length, GSI and HSI

No mortality was observed during the period of experiment. No difference in body mass, total length and HSI of VZ-treated fish were observed compared to solvent control or compared to E_2 treated group ($p > 0.05$) (Table 2). These parameters were also unchanged in E_2-treated group compared to solvent control ($p > 0.05$) (Table 2). In fish exposed to VZ, GSI was unchanged compared to solvent control ($p > 0.05$). However a tendency for a decrease of GSI was observed when VZ concentration was increased (Table 2), but no significant difference in GSI was observed between VZ-treated groups. Compared to fish exposed to E_2, GSI was higher in all VZ-treated groups ($p = 0.01$) (Table 2). Compared to solvent control, GSI was also lower in E_2-treated group ($p < 0.01$) (Table 2).

3.2. Sperm volume, motility and velocity

No sperm was produced in fish exposed to E_2. Sperm volume was reduced in fish exposed to VZ in a concentration-dependent manner, and a significant decrease was observed at 800 μg/L compared to control ($p < 0.05$, Fig. 1a). Sperm motility and velocity were evaluated at 15, 30, 45 and 60 s post activation and compared with solvent control. Sperm motility was decreased in fish exposed to 800 μg/L VZ evaluated at 15, 30 and 45 s post activation ($p < 0.01$) and in fish exposed to 400 and 800 μg/L VZ at 60 s post activation ($p < 0.001$, Fig. 1b). Sperm velocity evaluated at 15 and 30 s post activation was decreased in fish exposed to 800 μg/L VZ ($p < 0.01$), but it was unchanged across all treatments at 45 and 60 s post activation (Fig. 1c). In fish exposed to 400 and 800 μg/L VZ, spermatozoa without flagella or with damaged flagella were observed (Fig. 2).

3.3. Sex steroids (11-KT and E_2)

An increase and a decrease in 11-KT level were observed in fish exposed to 100 and 800 μg/L VZ compared to control, respectively ($p < 0.001$, Fig. 3a). 11-KT level in fish exposed to 100 and 400 μg/L VZ was higher than that of fish exposed to E_2 ($p < 0.01$, Fig. 3a). Increase in VZ concentrations resulted in 11-KT reduction; significant difference was observed between fish exposed to 100 and 800 μg/L VZ ($p < 0.001$, Fig. 3a). E_2 level was unchanged in VZ-treated groups compared to control ($p > 0.05$), but it was lower than in fish exposed to E_2 ($p < 0.001$, Fig. 3b). E_2 level in fish exposed to 100 μg/L VZ was higher than in those exposed to 400 and 800 μg/L VZ ($p < 0.05$, Fig. 3b).

4. Discussion

This study shows a decrease in sperm volume, and an impairment of sperm motility and velocity in goldfish exposed to VZ associated with alternations in testicular steroidogenesis. Complementary to the present results, our in vitro study showed no effect of VZ on sperm motility, when it was added into the activation

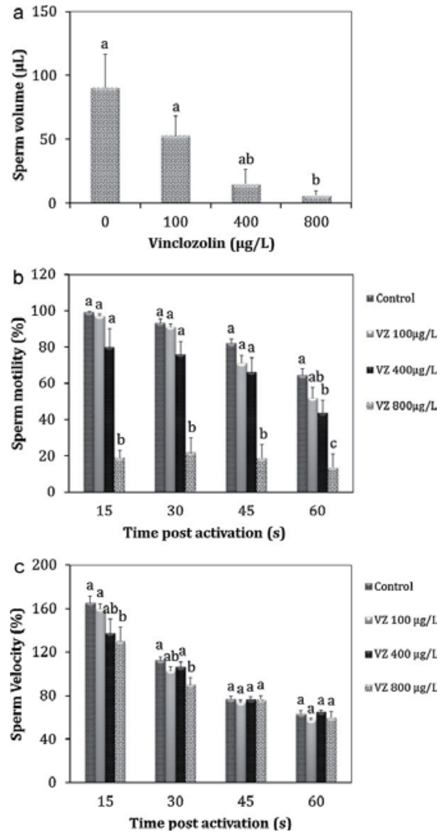

Fig. 1. Sperm volume (a), motility (b) and velocity (c) in male goldfish exposed to nominal concentrations of 100, 400, and 800 μg/L vinclozolin (VZ) for one month. Data are mean ± S.E.M. ($n = 6$). At each time post activation, values with different superscripts are significantly different.

medium up to 20×10^3 μg/L (Hatef et al., unpublished data). These data suggest that sperm quality and male fertility is one target for VZ effects, which disrupts endocrine regulation of reproduction in a concentration-dependent manner.

In this study, sperm volume decreased in fish exposed to 800 μg/L VZ. Similarly, reduction in sperm volume has been reported in rats administered with 100 mg/kg VZ (Quignot et al., 2012). Moreover, no sperm was produced in goldfish exposed to E_2. It has been already shown that E_2 reduces sperm volume to 50% compared to control in salmonids exposed to ≥1 ng/L for 35 days (Lahnsteiner et al., 2006). Sperm volume is determined by seminal plasma secretion and sperm release from testis into the spermatic duct. These phenomena are particularly regulated by 11-KT in fish. Therefore, observed decreases in 11-KT level in VZ 800 μg/L and E_2 groups suggest the hypothesis that both VZ and E_2 acts on testis causing reduction in sperm production by 11-KT biosynthesis disruption leading to partial or absolute inhibition of spermatogenesis. Previous studies have shown a decrease in sperm number when fish (Bayley et al., 2003) (Table 1) or mammals were exposed to VZ (Elzeinova et al., 2008; Eustache et al., 2009; Quignot et al., 2012).

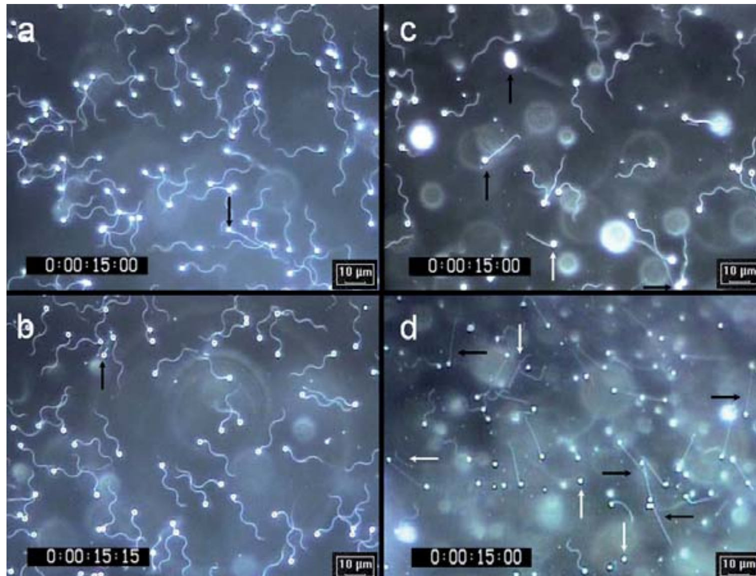

Fig. 2. Photographic images of goldfish sperm after activation in NaCl 50 mM, Tris 20 mM, pH 8.5 under darkfield microscope equipped with stroboscopic lamp. The images show sperm motility at 15 s post activation from control (a) and fish exposed to VZ at 100 µg/L (b), 400 µg/L (c) and 800 µg/L (d). Flagellar structure is beating in the motile sperm, while it is straight in immotile sperm (black arrows). In fish exposed to 400 and 800 µg/L VZ, spermatozoa with damaged flagella or without flagella were observed (white arrows).

To our knowledge, this is first study that shows a decrease of sperm motility and velocity in fish exposed to VZ. Adverse effects of VZ on sperm motility and velocity have been shown in mammals (Eustache et al., 2009). Evaluation of both sperm motility and velocity is a critical endpoint of EDCs on fertilizing ability of sperm, because male fertility is highly dependent on these parameters in fish (McAllister and Kime, 2003; Lahnsteiner et al., 2006; Linhart et al., 2008). Therefore, our results provide valuable reason for observed decrease in fertility of fathead minnow exposed to VZ (Martinovic et al., 2008). Lahnsteiner et al. (2006) reported sperm motility and fertility decrease in salmonids exposed to 1 ng/L E_2 for 35 days. Adverse effects of VZ or E_2 on sperm motility might be due to disruption in spermatozoa maturation, which is an important step for acquisition of potential for sperm activation (Morisawa and Morisawa, 1986; Alavi and Cosson, 2006) or damage to sperm cells (Hatef et al., 2011). In freshwater fish, including goldfish, sperm is immotile in the seminal plasma and sperm activation is induced by a hypo-osmotic signal, which triggers Ca^{2+}-dependent flagellar beating (Morisawa and Morisawa, 1986; Alavi and Cosson, 2006). In this regard, plasma membrane and molecular structure of sperm flagella are key targets for EDCs (Hatef et al., 2011). Similar to McAllister and Kime (2003), we observed morphological damages to spermatozoa such as sperm cells with cut flagella or without flagella. Until now, no information is available to show potential effects of EDCs via alternations in sperm maturation. In this context, studying luteinizing hormone (LH)-dependent progestagen biosynthesis in sperm cells, which regulate sperm maturation, needs to be considered in further studies (Miura et al., 1992; Nagahama, 1994). Decrease of sperm velocity in goldfish exposed to VZ (present study) or E_2 (Lahnsteiner et al., 2006) might be addressed to ATP content and regeneration of ATP; suggesting that mitochondria are targets of VZ (Hatef et al., 2011). Sperm velocity in fish is highly depending on flagellar beating, which is ATP-dependent activity (Alavi et al., 2009; Butts et al., 2010).

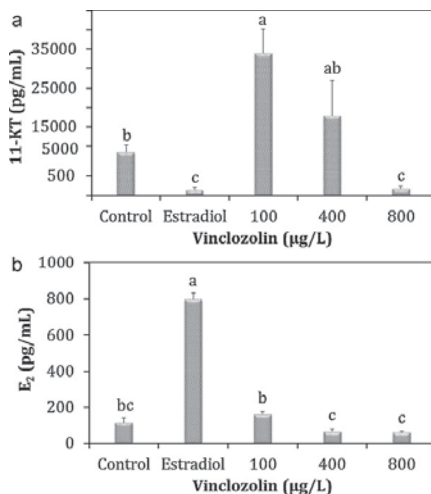

Fig. 3. 11-Ketotestosterone (11-KT, a) and 17β-estradiol (E_2, b) and levels in blood plasma of goldfish exposed to nominal vinclozolin (100, 400, and 800 µg/L) and 17β-estradiol (5 µg/L) concentration for one month. Data are mean ± S.E.M. (n = 6). Values with different superscripts are significantly different.

Endocrine disruption occurs when sex steroid concentrations are either high or low, or hormonal balance changes (Quignot et al.,

2012). In the present study, we evaluated 11-KT, because it is major androgen in fish, regulating spermatogenesis and being involved in sperm maturation (Borg, 1994; Young et al., 2005). Our results show significant concentration-dependent effects of VZ on steroidogenesis (11-KT and E_2). At lowest concentration examined (100 μg/L VZ), 11-KT increased in goldfish. This is consistent with previous studies in fathead minnow exposed to 400 and 700 μg/L (Martinovic et al., 2008) (Table 1). Similar trend in androgen (particularly T) level has frequently been reported in mammals (Kubota et al., 2003; Quignot et al., 2012). The physiological reasons for androgen elevation are unclear and remain to be investigated in further studies with emphasis on hypothalamic and pituitary functions. In this context, it has been hypothesized that VZ influences testicular functions via competitive binding to AR as antagonist action (Kelce et al., 1997; Martinovic-Weigelt et al., 2011). Due to anti-androgenic activity of VZ, it may decrease androgen production in testis, but information is very rare. For the first time, at least if fish species (Table 1), we have observed a decrease in 11-KT in fish exposed to 800 μg/L VZ, which shows similarities to other anti-androgens such as cyproterone acetate and bisphenol A (Sharpe et al., 2004; Hatef et al., 2012). Therefore, our data suggest potential VZ effect to influence testicular functions via inhibition of 11-KT. Following E_2 exposure for one month, 11-KT was also decreased in goldfish which is consistent with other studies (MacLatchy et al., 1997; Sharpe et al., 2007). These results suggest involvement of EDCs with both anti-androgenic and estrogenic modes of action in disruption of reproductive endocrine system by altering biosynthesis of androgens, particularly 11-KT which regulate spermatogenesis in fish.

Following exposure to 100 μg/L VZ, no change in E_2 level was observed compared to control, which is consistent with a previous study in fathead minnow (Martinovic et al., 2008) (Table 1). But, our study showed significant E_2 reduction in fish exposed to 400 and 800 μg/L compared to control and to 100 μg/L VZ. Eustache et al. (2009) also reported lower E_2 level in rats administrated with 30 compared to 1 mg/kg/bw VZ. Recently, a decrease of E_2 level was shown in rat administrated with 100 mg/kg VZ (Quignot et al., 2012). One study in fathead minnow shows significant increase of E_2 at 700 μg/L VZ (Makynen et al., 2000). The high variations among studies suggest uncertain potential impacts of VZ on E_2-mediated reproductive function. In this study, we showed an elevation in E_2 only in fish exposed to E_2, which confirms the estrogenic potency of E_2, when present at adequate levels in the aquatic environment, to disrupt reproduction (Bolger et al., 1998; Seki et al., 2006).

This study shows different effects of VZ and E_2 on GSI. Unchanged GSI in VZ treatments compare to control confirms previous studies in fathead minnow (Makynen et al., 2000) and in rats (Eustache et al., 2009; Quignot et al., 2012). There is one study that reports significant increase in GSI of fathead minnow exposed to 255 or 450 μg/L VZ (Martinovic et al., 2008) (Table 1). Elzeinova et al. (2008) reported no change and a decrease in GSI of mice at sexual differentiation and maturation stage, respectively. Therefore, VZ effect on GSI depends largely on the life stage of experimental animal used in different studies. In contrast, our data are consistent with several studies that show a reduction in GSI of fish exposed to E_2 (Kang et al., 2002; Seki et al., 2006; Sharpe et al., 2007). These findings suggest that GSI may not be the main endpoint for toxicity of VZ. Neither VZ nor E_2 affect HSI as shown in our work and previous studies (Kang et al., 2002; Eustache et al., 2009; Quignot et al., 2012).

In conclusion, this study shows that anti-androgen VZ disrupts testicular steroidogenesis in concentration-dependent manner, which causes reduction in sperm quality. The present study focused on testicular steroidogenesis and results showed 11-KT increase and decrease in fish exposed to 100 and 800 μg/L VZ, respectively. The observed decrease of 11-KT suggests direct effects of VZ on testicular function, similar to other anti-androgens. However, the mechanisms of action need to be investigated in further studies. However, an increase in 11-KT has been frequently reported, which suggests VZ effects via AR antagonism. Damages to sperm morphology observed in the present study suggest multiple targets for VZ to impair male fertility. Further studies need to investigate modulations in neuroendocrine control of steroidogenesis and sperm maturation for better understanding of VZ effects in fish reproduction.

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CHAPTER 4

SPERM IMPAIRMENTS ASSOCIATED WITH TRANSCRIPTOMIC ALTERNATIONS IN SEX STEROID RECEPTORS AND STEROIDOGENESIS GENES

4.1. Hatef, A., Zare, A., Alavi, S.M.H., Habibi, H.R., Linhart, O., 2012. Modulations in androgen and estrogen mediating genes and testicular response in male goldfish exposed to bisphenol A. Environmental Toxicology and Chemistry 31, 2069-2077.

4.2. Hatef, A., Alavi, S.M.H., Milla, S., Butts, I.A.E., Rodina, M., Carnevali, O., Fontaine, P., Linhart, O., 2012. Di-(2-ethylhexyl)-phthalate impairs sperm quality in goldfish associated with disruption in androgenesis. (submitted)

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MODULATIONS IN ANDROGEN AND ESTROGEN MEDIATING GENES AND TESTICULAR RESPONSE IN MALE GOLDFISH EXPOSED TO BISPHENOL A

AZADEH HATEF,*† AVA ZARE,‡ SAYYED MOHAMMAD HADI ALAVI,† HAMID R. HABIBI,‡ and OTOMAR LINHART†
†South Bohemia Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic
‡Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

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Abstract—Adverse effects of bisphenol A (BPA) on reproductive physiology were studied in male goldfish (*Carassius auratus*) exposed to nominal environmentally relevant concentrations (0.2 and 20 μg/L) for up to 90 d. Transcriptions of various reproductive genes were measured in brain, liver, and testis to investigate the BPA modes of action. Volume, density, total number, motility, and velocity of sperm were measured to assess testicular function. At 0.2 μg/L, BPA reduced steroidogenetic acute regulatory protein and increased estrogen receptors (ERs) messenger RNA (mRNA) transcript (ERβ1 in liver and ERβ2 in testis) after 90 d. At 20 μg/L, BPA increased mRNA transcript of androgen receptor in testis, brain- and testis-specific aromatase, and vitellogenin in liver after 90, 30, 60, and 60 d, respectively. Transcripts of ERs mRNA were increased after 30 to 60 d at 20 μg/L BPA; increase in ERβ1 mRNA was observed in testis after 7 d. Total number, volume, and motility of sperm were decreased in males exposed to 0.2 and 20 μg/L BPA, whereas sperm density and velocity were only reduced at 20 μg/L BPA. The results support the hypothesis that BPA may exert both anti-androgenic and estrogenic effects, depending on concentration, leading to diminished sperm quality. The findings provide a framework for better understanding of the mechanisms mediating adverse reproductive actions of BPA observed in different parts of the world. Environ. Toxicol. Chem. 2012;31:2069–2077. © 2012 SETAC

Keywords—P450 aromatase Sex steroid receptor Sperm Steroidogenic acute regulatory protein Vitellogenin

INTRODUCTION

Bisphenol A (BPA) is widely used for production of polymers such as polycarbonates, epoxy, phenolic resins, polyesters, and polyacrylates to manufacture polycarbonate plastic products, resins lining cans, dental sealants, and food industry products, such as containers, plastic wrappers, and inner coatings for cans [1,2]. Studies in mammals demonstrated that BPA exerts both estrogenic and anti-androgenic effects, depending on concentration and exposure time [2–4]. In fish, most studies showed estrogenic effects of BPA such as stimulation of vitellogenin (Vtg) and transcription alternations of specific genes in different organs [5,6]. However, the adverse effects of BPA at environmentally relevant concentrations are still unclear on reproductive performance in fish. Moreover, BPA molecular modes of action are still unknown because transcription alternations have been studied in a few numbers of genes and have not been compared between organs involved in reproduction, including brain, liver, and gonad.

Synthesis of Vtg in the liver is induced by 17β-estradiol (E_2) [7]. Although the Vtg gene is present in the liver of both sexes, it is only elevated in females because of endogenous estrogens. In males, Vtg is low and only becomes detectable when they are exposed to estrogen or xenoestrogens. Therefore, Vtg has been used for the identification of estrogenic-like contaminants [5,8,9]. Induction of Vtg gene and its synthesis has been shown in a concentration- and time-dependent manner in different fish species exposed to BPA at usually high concentrations (higher than 100 μg/L) after two to four weeks of exposure [10–13]. The present study investigated whether lower BPA concentrations approaching environmental levels alter Vtg messenger RNA (mRNA) transcript in liver.

The physiological functions of estrogen receptors (ERs) in E_2-mediated induction of Vtg have been investigated in goldfish [14,15], demonstrating direct involvement of ERβ subtypes in Vtg synthesis as well as the induction of ERα. Several studies show modulations in both ERα and ERβ in mammals administered by BPA [16–19], but BPA affinity to ERs is unknown in fish. Additionally, BPA effects on aromatase P450 in brain (CYP19b) and testis (CYP19a), which mediates conversion of androgens into estrogens [20,21], were studied in the present study. Results of ERs and P450 aromatase provide valuable information about the mechanism in which normal E_2-mediated Vtg synthesis is disrupted by BPA.

Decrease of androgens has been previously reported in fish exposed to BPA at low (0.6–11 μg/L) [22] or high (1,000 μg/L) [13] concentrations, but mechanisms of action are still unknown. Studies show a decrease of androgens mediated by luteinizing hormone secretion from pituitary via androgen receptor (AR) antagonist [19,23,24]. Sharpe et al. [25] suggested that a decrease in androgens occurs via down-regulation of steroidogenic acute regulatory protein (StAR) that delivers cholesterol to the inner mitochondrial membrane in male goldfish exposed to β-sitosterol. In the present study, AR and StAR modulations were measured for understanding of BPA effects in decreasing androgens.

Two studies show adverse effects of BPA on sperm production and motility in fish [22,26]. These studies revealed delays in spermiation and decrease in sperm quality in males exposed to BPA at environmentally relevant concentrations. In the present study, sperm quality was also investigated after 90 d exposure to BPA to investigate the correlation between mRNA transcript of reproductive genes and change in sperm quality.

* To whom correspondence may be addressed (ahatef@frov.jcu.cz).
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Goldfish were exposed to BPA at 0.2 and 20 μg/L, which overlaps with a range of concentrations reported in the European rivers [3].

MATERIAL AND METHODS

Experimental design

Mature male goldfish (*Carassius auratus*) (2- to 3-year-old) were obtained from Hluboka fish farm, Hluboka nad Vltavou, Czech Republic, at the beginning of the spawning season. The health status of fish was first checked by random selection of some individuals. Neither parasite nor disease was detected in their gills or mucus. Then, they were acclimatized and distributed into the aquaria. All groups, including control and BPA-treated groups, were under similar condition; each group was kept in 70-L aquaria and maintained under a 12-h light/12-h dark photoperiod. To be closer to physiological condition, water in the aquaria was not heated, and temperature was measured as 18 and 22°C at the beginning and end of the experiment, respectively. Water dissolved oxygen and pH were checked three times per week before feeding and were 6.0 mg/L (5.7–6.2) and 7.5 (7.3–7.8), respectively. The fish were fed once per day with commercial food (ZZN Vodnany).

Fish were exposed to BPA (Sigma-Aldrich) in May 2009. Duplicate tanks of adult male goldfish were exposed to nominal 0.2 and 20 μg/L BPA and solvent control for a period of 90 d. Dimethyl sulfoxide (Sigma-Aldrich) was used to dissolve BPA, and its final concentration was 0.001% in each aquarium. A control group without dimethylsulfoxide was also studied, but the results did not show any significant difference from solvent control (see Tables 1 and 2, and Fig. 1). Therefore, only the data from solvent control corresponding to each sampling time were used as reference for comparison with respective treatments. Every other day, 80% of the exposure solution was renewed.

Thirty individuals were introduced to each aquarium to collect five samples at 7, 15, 30, and 60 d and 10 samples at 90 d after exposure. Sample size differences in the present study were attributable to the existence of female individuals. This was because of no sexual character to recognize males and females. Samples were collected from at least three individuals at each sampling time. At each sampling time, fish were first anesthetized in 2-phenoxyethanol dissolved in water (0.3 ml/L). At 90 d after exposure, sperm collection for quality assessment (methods outlined later) was performed before sampling of organs. Each male was individually weighted (± 0.1 g) and measured for total length (± 1 mm). Fish were then sacrificed by cutting spinal cord; testis and liver were removed and weighed (± 0.01 g) for determination of gonadosomatic index (GSI) (= 100 × gonad wt/total fish wt) and hepatosomatic index (HSI) (= 100 × liver wt/total fish wt). Samples were immediately frozen in liquid nitrogen and kept in an RNAse-free tube at −80°C until use.

Table 2. Sperm volume, sperm density, and total number of sperm cells in male goldfish (*Carassius auratus* L.) exposed to different concentrations of bisphenol A (BPA) after 90 d exposure[a]

Parameter	Sperm volume μl	Sperm density × 10^9 cells/ml	Total number of spermatzoa × 10^9 cells
Control	195.0 ± 15.3[a]	13.2 ± 0.8[a]	2.7 ± 0.1[a]
Solvent control	207.1 ± 60.8[a]	12.9 ± 2.7[a]	2.6 ± 0.7[a]
0.2 μg/L BPA	52.9 ± 25.0[b]	11.1 ± 1.4[a,b]	0.8 ± 0.3[b]
20 μg/L BPA	58.8 ± 26.7[b]	9.2 ± 1.4[b]	0.9 ± 0.4[b]

[a] Solvent control group was exposed to 0.001% dimethylsulfoxide (DMSO) ($n = 8$).
[b] At each column, values with different superscripts are significantly different ($p < 0.05$).

RNA extraction and complementary DNA synthesis

Total RNA was extracted from each tissue sample of male fish, using Trizol Reagent (Invitrogen, Cat. No. 15596-018), following the manufacturer's instructions. Total RNA concentration was estimated from absorbance at 260 nm (A260 nm,

Table 1. Body mass (g), total length (cm), gonadosomatic index (GSI, %), and hepatosomatic index (HSI, %) of male goldfish (*Carassius auratus* L.) exposed to different concentrations of bisphenol A (BPA) sampled at 7, 15, 30, 60, and 90 d after exposure[a,b]

		7 d		15 d		30 d		60 d		90 d	
Parameter	BPA (μg/L)	*n*	Measurement	*n*	Measurement	*n*	Measurement	*n*	Measurement	*n*	Measurement
Body mass											
	Control	4	49.4 ± 6.4	4	48.8 ± 6.6	4	54.0 ± 6.2	4	38.1 ± 11.1	8	38.9 ± 3.7
	Solvent control	5	44.1 ± 6.9	4	48.5 ± 7.9	3	50.0 ± 8.7	4	36.6 ± 6.1	8	38.1 ± 7.4
	0.2	3	49.0 ± 13.5	4	45.0 ± 4.6	4	46.3 ± 5.5	5	55.4 ± 8.9	8	28.1 ± 4.7
	20	4	39.4 ± 5.9	3	45.0 ± 2.9	3	45.0 ± 5.8	4	47.2 ± 7.5	8	34.4 ± 4.9
Total length											
	Control	4	14.1 ± 0.8	4	14.1 ± 0.6	4	14.2 ± 1.1	4	12.6 ± 1.4	8	13.5 ± 2.8
	Solvent control	5	13.5 ± 0.8	4	13.3 ± 1.1	3	14.5 ± 0.8	4	12.7 ± 0.8	8	12.4 ± 0.8
	0.2	3	13.7 ± 1.6	4	13.5 ± 0.6	4	13.7 ± 0.9	5	14.1 ± 1.1	8	11.1 ± 0.5
	20	4	11.9 ± 1.4	3	13.9 ± 0.6	3	13.5 ± 0.8	4	14.0 ± 1.1	8	11.8 ± 0.5
GSI											
	Control	4	2.5 ± 0.9	4	4.4 ± 0.8	4	3.5 ± 1.4	4	3.1 ± 1.1	8	3.1 ± 1.1
	Solvent control	5	3.8 ± 1.2	4	3.5 ± 0.5	3	3.4 ± 0.4	4	2.7 ± 0.8	8	4.0 ± 0.5
	0.2	3	2.1 ± 0.8	4	3.1 ± 1.0	4	3.3 ± 0.9	5	3.9 ± 1.2	8	2.9 ± 0.3
	20	4	3.9 ± 0.7	3	4.3 ± 1.2	3	1.9 ± 0.7	4	3.0 ± 0.5	8	3.1 ± 0.5
HSI											
	Control	4	3.6 ± 1.1	4	3.8 ± 0.6	4	3.8 ± 0.4	4	3.5 ± 0.3	8	2.8 ± 0.7
	Solvent control	5	3.9 ± 0.6	4	2.6 ± 0.3	3	3.3 ± 0.7	4	3.8 ± 0.3	8	3.3 ± 0.5
	0.2	3	2.8 ± 0.1	4	3.6 ± 0.6	4	3.1 ± 0.1	5	3.2 ± 0.2	8	3.3 ± 0.2
	20	4	4.9 ± 0.8	3	3.7 ± 0.2	3	4.5 ± 0.7	4	4.1 ± 0.5	8	3.1 ± 0.2

[a] Solvent control group was exposed to 0.001% dimethylsulfoxide (DMSO).
[b] No significant differences were observed between groups at each sampling time ($p > 0.05$).

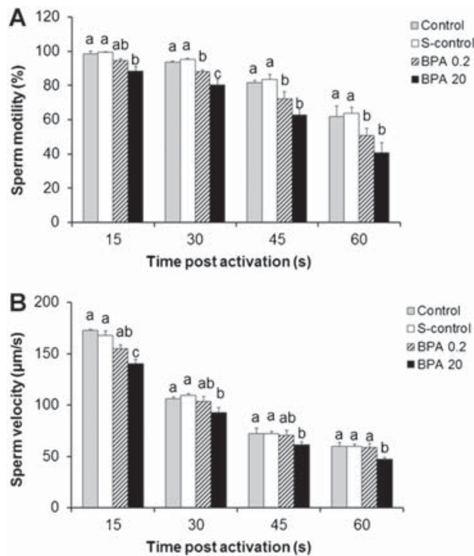

Fig. 1. Sperm motility (**A**) and velocity (**B**) in male goldfish exposed to bisphenol A (BPA). The fish were exposed to nominal 0.2 and 20 µg/L BPA and sperm samples were collected at 90 d following exposure. Data represents mean ± SEM. Values with different superscripts are significantly different for sperm volume, density and total number of spermatozoa (n = 8, $p < 0.05$). In case of sperm motility and velocity, values with different superscripts are significantly different at each time post activation ($n = 8$, $p < 0.05$).

Nanodrop, USA), and RNA quality was verified by A260 nm/A280 nm ratios between 1.8 and 2 and A230 nm/A260 nm ratios greater than 2. Complementary DNA was synthesized from 4 µg total RNA of each sample using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) (Invitrogen, Cat No. 28025-013) and oligo $(dT)_{18}$ primer (Promega) following the manufacturer's instructions. Briefly, 2 µl Oligo $(dT)_{18}$ primer (500 µg/ml) was added to each sample and the reaction mixture was heated to 70°C for 10 min and then quickly chilled to 4°C. After cooling, 4 µl of 5× first-strand buffer, 2 µl 100 mM dithiothreitol, 0.4 µl deoxyribonucleotide (dNTP; 100 mM; cat num:dNTP-01,UBI Life Science) and 0.7 µl M-MLV (200 U/µl) were added to a total volume of 18 µl. The reaction mixture was then incubated at 25°C for 10 min and at 37°C for 50 min using an iQ cycler. By heating at 70°C for 15 min, the reaction was deactivated. Each 18-µl reaction was diluted threefold in nuclease-free water and used as a template for quantitative real-time polymerase chain reaction (PCR) assay.

Quantitative real-time PCR

The iCycler iQ Real-time PCR Detection System (Bio-Rad Laboratories) was used for evaluating gene expression level with the following condition per reaction: 1 µl diluted complementary DNA, 0.26 µM each primer, 12.5 µl SYBR Green PCR Master Mix(Qiagen), and ultrapure distilled water (Invitrogen) to a total volume of 25 µl. Specific primers were used to detect expression of different genes (Table 3) [15]. The thermal profile for all reactions was 3 min at 95°C and then 45 cycles of 10 s at 95°C, 20 s appropriate annealing temperature and 72°C. The specificity of the amplified product in the quantitative PCR assay was determined by analyzing the melting curve to discriminate target amplicon from primer dimer or other nonspecific products. A single melt curve was observed for each primer set in all quantitative PCR reactions. Each individual sample was run in triplicate, and the mean threshold cycles (as determined by the linear portion of the fluorescence absorbance curve) were used for the final calculation. The mRNA expression levels were normalized to the expression level of glyceraldehyde-3- phosphate dehydrogenase mRNA, using the standard $2^{-\Delta\Delta Ct}$ method.

Sperm quality assessment

Although our previous study showed the effects of BPA on sperm quality [22], confirming the decrease in sperm quality in the present study was important. Therefore, sperm quality was evaluated only once, following 90 d exposure. Sperm was collected from each individual by a gentle abdominal massage from the anterior portion of the testis toward the genital papilla and collected with plastic syringes and kept on ice (2−4°C) until analysis. Attention was focused on avoiding sperm contamination by urine, mucus, blood, and water.

Sperm volume and density were measured and expressed as milliliters and milliard of sperm per milliliter, respectively. Sperm density was measured by counting, following the method of Alavi et al. [27]. Semen was diluted two times, each 100-fold with 0.7% NaCl. Ten microliters diluted semen was placed onto a haemocytometer covered with a coverslip and left for 10 min to allow sperm sedimentation before 16 cells (0.1-mm depth and 0.2 length) were counted. Total number of spermatozoa is recounted as spermatozoa concentration × sperm volume.

Table 3. Primer sequences and amplification (annealing) programs used for measurements of selected genes using qRT-PCR

	Forward primer	Reverse primer	Annealing temperature (°C)
StAR	ATGGCTGGCAAACTGAGATCGAGA	TCCATGTTATCCACCAGCTCCTCA	57
CYP19a	TTGTGCGGGTTTGGATCAATGGTG	TTCCGATACACTGCAGACCCAGTT	55
CYP19b	AGGCGAGCGGGATGTAGAGT	CGTCCGATGTTCAGGATGAGG	58
AR	GATGAAAGGTCCAGAGGAGG	ACTGTGAGTGGAACGTCAGG	55
ERα	GAGGAAGAGTAGCAGCACTG	GGCTGTGTTTCTGTCGTGAG	55
ERβ1	GGCAGGATGAGAACAAGTGG	GTAAATCTCGGGTGGCTCTG	55
ERβ2	GGATTATTCACCACCGCACG	TTCGGACACAGGAGGATGAG	55
Vtg	GAAGTGCGCATGGTGGCTTGTATT	AGCTGCCATATCAGGAGCAGTGAT	55
GAPDH	TGATGCTGGTGCCCTGTATGTAGT	TGTCCTGGTTGACTCCCATCACAA	57

qRT-PCR = quantitative real-time polymerase chain reaction; StAR = steroidogenic acute regulatory protein; CYP19a = gonad aromatase; CYP19b = brain aromatase; AR = androgen receptor; ERα, ERβ1, and ERβ2 = estrogen receptor subtypes; Vtg = vitellogenin; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

To evaluate the sperm motility traits (percentage of sperm motility and sperm velocity), sperm of each individual was directly activated in NaCl 50 mM, KCl 5 mM, Tris 20 mM, pH 8.5, 110 mOsmol/kg at ratio 1:1,000–2,000 (sperm:activation solution). To avoid sperm stickness into the slides, bovine serum albumin was added into the activation medium right before adding sperm at a final concentration of 0.1% w/v. Sperm motility was recorded using a CCD video camera (SONY DXC-970MD) mounted on a darkfield microscope (Olympus BX50) supplied with a stroboscopic lamp and a DVD-recorder (SONY DVO-1000 MD). Then, the five successive frames were captured and analyzed with a micro image analyzer (Olympus Micro Image 4.0.1. for Windows) to measure sperm motility (%) and velocity (μm/s) based on successive position of sperm heads (for details see Hatef et al. [28]). For each individual, three separate records were performed, and the mean of the values was used in statistical analysis. Data of sperm velocity presented in results are from only motile spermatozoa. For each record, sperm velocities were measured in 30 to 40 spermatozoa, and mean values were used in statistical analysis.

Statistical analysis

All data were analyzed using SPSS 9.0 for Windows, and each male was considered a replicate. Data for AR, ER subtypes, P450 aromatase, and Vtg were log transformed to meet assumptions of normality and homoscedasticity examined by Levene's test. Repeated-measure analysis of variance (ANOVA; alpha at 0.05) was first used to understand the effects of main factors; BPA concentration, exposure time, and their interaction [24,32]. Results were summarized as $F_{\text{df, ddf}}{}^{P}$, where F is F value, df is degree of freedom, ddf is error of df, and p is p value. To understand the effects of BPA concentrations, the model was then revised into one-way ANOVA models followed by Tukey's post-hoc test at each sampling time (7, 15, 30, 60, or 90 d); either significant (ERβ1 and ERβ2 in liver) or nonsignificant (remained parameters) effects of main factors, and their interactions were observed. In case of sperm parameters, only one main factor was present (BPA concentration); therefore, one-way analysis of variance was performed to understand the effects of BPA on sperm parameters. All data are presented as mean ± standard error of mean.

RESULTS

No mortality was observed in any treatments or sampling time. Body mass and total length remained unchanged between the BPA-treated groups and controls (Table 1). Neither GSI nor HSI was affected by BPA concentration and exposure time (Tables 1 and 4).

StAR mRNA transcript

Exposure to BPA significantly influenced StAR mRNA transcript in the testis (Table 4). A decrease in StAR mRNA transcript was observed after 90 d exposure of males to 0.2 μg/L BPA ($F_2 = 6.3$, $p < 0.01$, Fig. 2A).

AR mRNA transcript

Transcript of AR mRNA in the testis was influenced by BPA concentration and exposure time (Table 4). The AR mRNA transcript was significantly lower in fish exposed to 0.2 μg/L BPA compared with those of 20 μg/L after 7 ($F_2 = 2.1$) and 15 d ($F_2 = 2.5$) exposure ($p = 0.05$), but the changes were not statistically different from those of solvent control (Fig. 2B). After 90 d exposure, AR mRNA transcript was increased at 20 μg/L BPA ($F_2 = 6.8$, $p < 0.01$, Fig. 2B).

Aromatase mRNA transcript

Both BPA concentration and exposure time significantly influenced CYP19b and CYP19a mRNA transcript in the brain and testis (Table 4). Increase in CYP19b mRNA transcript was observed in fish exposed to 20 μg/L BPA after 30 ($F_2 = 6.0$, $p < 0.05$) and 60 d ($F_2 = 11.4$, $p < 0.01$) exposure (Fig. 3A). In the testis, CYP19a mRNA transcript was increased after 60 ($F_2 = 2.5_3$, $p < 0.05$) and 90 d ($F_2 = 6.1$, $p < 0.01$) in fish exposed to 20 μg/L BPA (Fig. 3B).

Table 4. Summary of statistics ($F_{\text{df, ddf}}$) obtained from repeated-measures analysis of variance (ANOVA) models used to study the effects of main factors (bisphenol A [BPA] concentration, exposure time, and their interaction) on reproductive performances in male goldfish (*Carassius auratus* L.)

Selected genes	Organ	BPA concentration	Exposure time	BPA concentration × exposure time
GSI		$0.23_{2,73}$	$0.87_{4,73}$	$0.82_{8,73}$
HSI		$1.31_{2,73}$	$2.05_{4,73}$	$1.21_{8,73}$
StAR	Testis	$9.82_{2,40}^{***}$	$1.7_{4,40}$	$0.37_{8,40}$
AR	Testis	$4.63_{2,45}^{**}$	$2.9_{4,45}^{*}$	$0.85_{8,45}$
CYP19	Brain	$9.01_{2,42}^{***}$	$3.10_{4,42}^{*}$	$2.03_{8,42}$
	Testis	$10.8_{2,45}^{***}$	$3.4_{4,45}^{*}$	$1.11_{8,45}$
ERα	Brain	$5.8_{2,38}^{**}$	$2.2_{4,38}$	$0.82_{8,38}$
	Testis	$2.5_{2,45}$	$0.6_{4,45}$	$0.20_{8,45}$
	Liver	$0.4_{2,46}$	$3.1_{4,46}^{*}$	$1.24_{8,46}$
ERβ1	Brain	$3.8_{2,40}^{*}$	$3.8_{4,40}^{**}$	$1.25_{8,40}$
	Testis	$10.1_{2,39}^{***}$	$1.8_{4,39}$	$0.88_{8,39}$
	Liver	$9.3_{2,40}^{***}$	$6.0_{4,40}^{**}$	$1.88_{8,40}^{*}$
ERβ2	Brain	$4.4_{2,41}^{*}$	$2.7_{4,41}^{*}$	$1.01_{8,41}$
	Testis	$10.4_{2,41}^{***}$	$2.4_{4,41}^{*}$	$0.89_{8,41}$
	Liver	$16.6_{2,45}^{***}$	$2.7_{4,45}^{*}$	$2.29_{8,45}^{*}$
Vtg	Liver	$6.2_{4,33}^{**}$	$2.9_{2,33}^{*}$	$2.15_{8,33}$

$^{*}p < 0.05$.
$^{**}p < 0.01$.
$^{***}p < 0.001$.
GSI = gonadosomatic index; HIS = hepatosomatic index; StAR = steroidogenic acute regulatory protein; AR = androgen receptor; CYP19 = P450 aromatase; ERα, ERβ1, and ERβ2 = estrogen receptor subtypes; Vtg = vitellogenin.

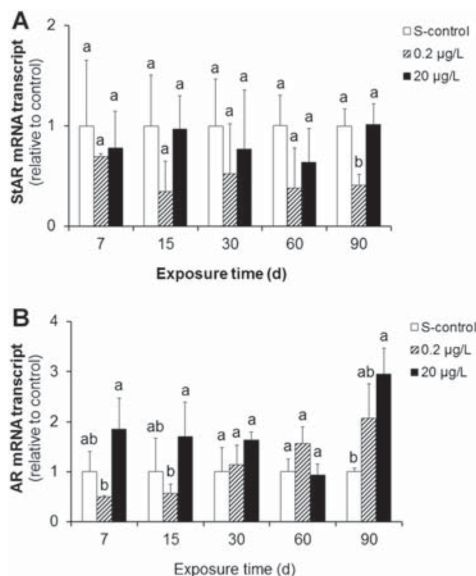

Fig. 2. Mean ± SEM messenger RNA (mRNA) transcript of steroidogenic acute regulatory protein (StAR) (**A**) and androgen receptor (AR) (**B**) in testis of male goldfish exposed to bisphenol A (BPA). The fish were exposed to nominal 0.2 and 20 μg/L BPA and transcript abundance is expressed relative to that of solvent control (0.001% dimethylsulfoxide [DMSO]). At each exposure time, values with different superscripts are significantly different ($n = 3–8$, $p < 0.05$).

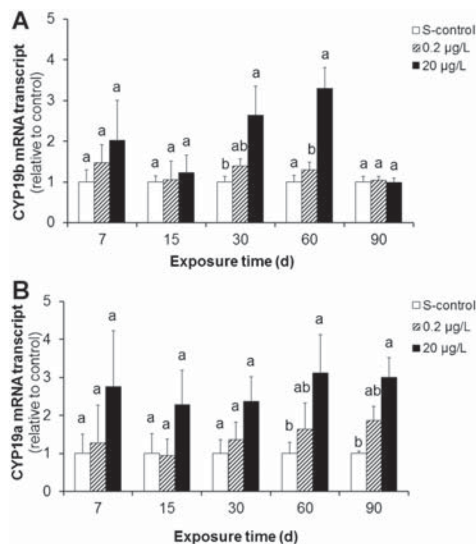

Fig. 3. Mean ± SEM messenger RNA (mRNA) transcript of P450 aromatase in brain (CYP19b) (**A**) and testis (CYP19a) (**B**) of male goldfish exposed to bisphenol A (BPA). The fish were exposed to nominal 0.2 and 20 μg/L BPA and transcript abundance is expressed relative to that of solvent control (0.001% dimethylsulfoxide [DMSO]). At each exposure time, values with different superscripts are significantly different ($n = 3–8$, $p < 0.05$).

ER subtypes mRNA transcript

Transcript of ERα mRNA was affected by BPA concentration in the brain and by exposure time in the liver (Table 4). Increase in the brain ERα mRNA transcript was observed after 30 ($F_2 = 8.1$) and 60 d ($F_2 = 2.0$) exposure to 20 μg/L BPA ($p < 0.01$, Fig. 4). In the testis and liver, the ERα mRNA transcript was unchanged (data are not shown).

Transcript of ERβ1 mRNA was affected by BPA concentration in the brain, liver, and testis, as well as exposure time in the brain and liver (Table 4). In the brain, increase in ERβ1 mRNA transcript was observed after 60 d exposure to 20 μg/L ($F_2 = 1.8$, $p < 0.05$, Fig. 5A). In the testis, ERβ1 mRNA transcript was increased at all time points after exposure to 20 μg/L; 7 d ($F_2 = 3.9$), 15 d ($F_2 = 4.9$), 30 d ($F_2 = 3.6$), 60 d ($F_2 = 1.8$), and 90 d ($F_2 = 4.2$) ($p < 0.05$, Fig. 5B). In the liver, ERβ1 mRNA transcript was increased at 60 d ($F_2 = 3.7$, $p < 0.05$) and 90 d ($F_2 = 8.4$, $p < 0.01$) in fish exposed to 20 μg/L and after 90 d exposure to 0.2 μg/L ($F_2 = 2.8$, $p < 0.05$) (Fig. 5C).

Both BPA concentration and exposure time affected ERβ2 mRNA transcript in the brain, liver, and testis (Table 4). Significant effect of BPA concentration and exposure time interaction was only observed in the liver (Table 4). In the brain, ERβ2 mRNA transcript was increased in fish exposed to 20 μg/L BPA after 30 d exposure ($F_2 = 7.0$, $p < 0.01$) (Fig. 6A). In the testis, ERβ2 mRNA transcript was increased in fish exposed to 20 μg/L BPA after 60 d ($F_2 = 2.1$, $p < 0.05$) and to 0.2 and 20 μg/L BPA after 90 d exposure ($F_2 = 15.7$, $p < 0.001$, Fig. 6B). In liver, an increase in ERβ2 mRNA transcript was observed after 60 ($F_2 = 5.05_3$) and 90 d ($F_2 = 7.7$) exposure to 20 μg/L BPA ($p < 0.01$, Fig. 6C).

Vtg mRNA transcript

The BPA concentration and exposure time influenced Vtg mRNA transcript in the liver (Table 4). An increase in Vtg mRNA transcript was observed after 60 ($F_2 = 4.3$) and 90 d ($F_2 = 5.1$) in fish exposed to 20 μg/L BPA ($p < 0.05$, Fig. 7).

Sperm quality

Sperm volume ($F_2 = 4.7$) and total number of spermatozoa ($F_2 = 3.2$) were decreased in both groups exposed to 0.2 and 20 μg/L BPA ($p < 0.05$, Table 2). Sperm density was decreased in males exposed to 20 μg/L BPA ($F_2 = 2.3$, $p < 0.05$, Table 2). Sperm motility was decreased in fish exposed to 20 μg/L ($F_2 = 20.6$, $p < 0.001$) evaluated at 15 s post activation and in both 0.2 and 20 μg/L BPA evaluated at 30 s ($F_2 = 32.2$, $p < 0.001$), 45 s ($F_2 = 11.2$, $p < 0.001$), and 60 s ($F_2 = 5.2$, $p < 0.01$) after activation (Fig. 1A). Sperm velocity showed a significant decrease in fish exposed to 20 μg/L BPA evaluated at 15 s ($F_2 = 10.5$, $p < 0.001$), 30 s ($F_2 = 4.1$, $p < 0.05$), 45 s ($F_2 = 3.3$, $p < 0.05$), and 60 s ($F_2 = 6.9$, $p < 0.01$) post activation (Fig. 1B).

DISCUSSION

For better understanding of the BPA mechanism of toxicity leading to reproductive endocrine disruption, the present study was performed to investigate correlation between sperm quality and mRNA transcript levels of genes involved in testicular function in goldfish. This study was carried out as a follow-up to our previous observation that exposure to BPA results in a decline in sperm quality associated with a decrease in androgens

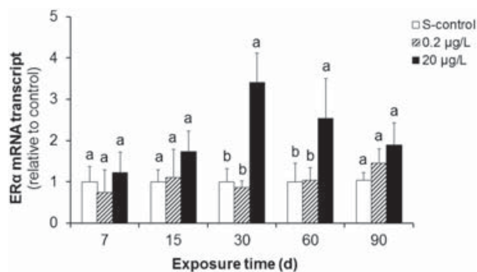

Fig. 4. Mean ± SEM messenger RNA (mRNA) transcript of estrogen receptor (ERα) in the brain of male goldfish exposed to bisphenol A (BPA). The fish were exposed to nominal 0.2 and 20 μg/L BPA and transcript abundance is expressed relative to that of solvent control (0.001% dimethylsulfoxide [DMSO]). At each exposure time, values with different superscripts are significantly different ($n = 3–8$, $p < 0.05$).

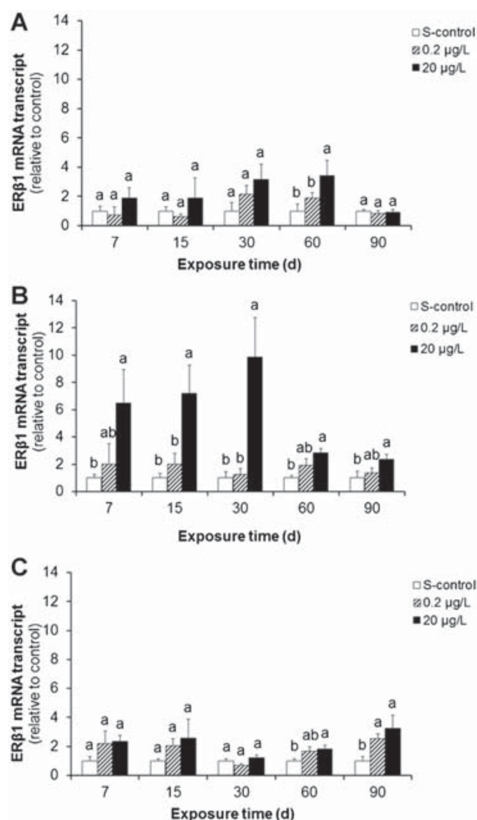

Fig. 5. Mean ± SEM messenger RNA (mRNA) transcript of estrogen receptor (ERβ1) in brain (**A**), testis (**B**) and liver (**C**) of male goldfish exposed to bisphenol A (BPA). The fish were exposed to nominal 0.2 and 20 μg/L BPA and transcript abundance is expressed relative to that of solvent control (0.001% dimethylsulfoxide [DMSO]). At each exposure time, values with different superscripts are significantly different ($n = 3–8$, $p < 0.05$).

[22]. The results demonstrate changes in mRNA transcripts of sex steroids, Vtg, and steroidogenic-mediating genes encoding steroidogenic enzymes in goldfish after exposure to 0.2 and 20 μg/L BPA. At 0.2 μg/L, StAR mRNA transcript was reduced in the testis and mRNA transcript of ERβ1 in the liver, and ERβ2 in the testis were increased after 90 d exposure (Table 5). At 20 μg/L, transcript of P450 aromatase, ERs, Vtg, and AR mRNA were increased after 30, 60, or 90 d exposure (Table 5). The BPA concentrations examined in the present study are relevant to the levels observed in the European rivers [3,6].

No mortality was observed in BPA-treated groups, indicating that the examined BPA concentrations were sublethal. The GSI and HSI were unchanged, which are consistent with previous works on goldfish, common carp, and medaka exposed to 0.6 to 11, 1 to 1,000 and 3,120 μg/L BPA for 30, 15, and 21 d, respectively [11,13,22,29]. Similarly, testis weight was also found to remain unchanged in mammals [18,19,30]. These findings suggest that GSI and HSI are not good indicators for screening adverse effects of BPA on fish reproduction.

The increase in AR mRNA transcript observed at 20 μg/L was concurrent with our previous results showing reduction in androgen production in goldfish exposed to 11 μg/L BPA [22]. A decrease in StAR mRNA transcript was observed at each time point examined, but the significant decrease was only observed after 90 d exposure to 0.2 μg/L BPA. This is because of high variations resulting from inter-animal variability as well as sample size. The observed decrease of StAR, in part, might be responsible for the reported decrease in androgen synthesis due to disruption of cholesterol transport to mitochondria. Similarly, Sharpe et al. [25] reported a decrease in StAR and androgen concentration in goldfish exposed to β-sitosterol after five months of exposure. Moreover, inhibition of androgen production interferes with a decrease of luteinizing hormone secretion in mammals via inhibition of 17α-hydroxylase [19,24]. These observations collectively suggest dose-dependent BPA modes of action to disrupt androgen biosynthesis in testis. At low doses, inhibition of androgen synthesis occurs via disruption of pituitary and testis functions to inhibit luteinizing hormone secretion or through a decrease in substrate availability mediated by StAR. At high doses, BPA acts through competitive binding to AR, which in turn suppresses luteinizing hormone function to stimulate androgen biosynthesis [19,24].

Exposure to 0.2 μg/L BPA stimulated mRNA transcripts of ERβ1 in liver and ERβ2 in testis after 90 d, but these modulations did not lead to an increase in Vtg mRNA transcript. The observed increase might be attributable to the affinity of BPA to ERs. Exposure to 20 μg/L BPA stimulated all three subtypes of ER in brain, liver, or testis, which led to an increase in Vtg mRNA transcript. The observed increase in ERs by BPA is consistent with the presumed estrogenic mimicking action [14,15]. Similarly, BPA increased ERα and ERβ1 mRNA transcript in tilapia [31]. Our results are also consistent with the report that in the brain, liver, and testis of a hermaphroditic fish; exposure to 600 μg/L BPA for 4 d increased ERα expression, whereas ERβ mRNA transcript remained unchanged [32]. Differences in BPA-induced ERs mRNA transcript might be attributable to species variability, maturity stage of fish, and concentration or period of exposure [4]. In the rat brain, BPA stimulated ERβ mRNA transcript without affecting that of ERα [19]. In the rat testis, BPA decreased and increased ERβ and ERα mRNA transcripts, respectively [19]. One study in male rats demonstrated an increase in both ERβ and ERα mRNA transcript in the brain, probably because of high concentration

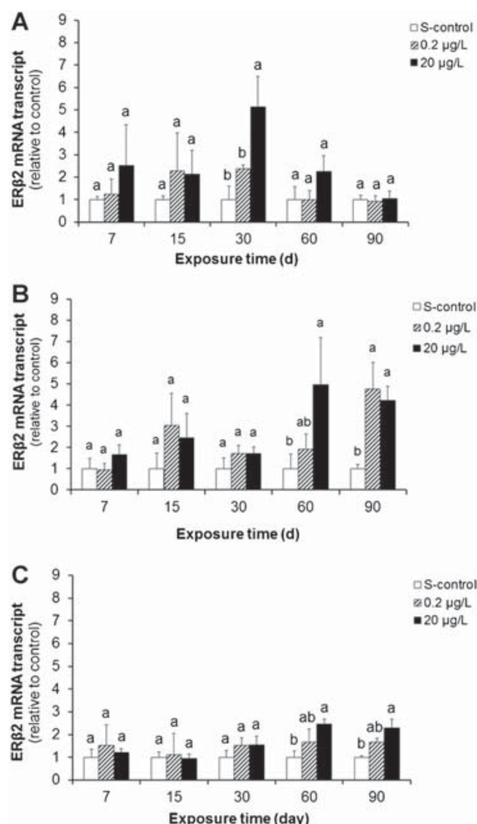

Fig. 6. Mean ± SEM messenger RNA (mRNA) transcript of estrogen receptor (ERβ2) in brain (**A**), testis (**B**) and liver (**C**) of male goldfish exposed to bisphenol A (BPA). The fish were exposed to nominal 0.2 and 20 μg/L BPA and transcript abundance is expressed relative to that of solvent control (0.001% dimethylsulfoxide [DMSO]). At each exposure time, values with different superscripts are significantly different ($n = 3–8$, $p < 0.05$).

of BPA (100 μg/kg body wt per day). Contributing factors that may explain observed variability in ERs' response to BPA include differences in exposure time, concentration, and mode of administration as well as differences in affinity of BPA to various ERs [15,17,19,33]. The present results demonstrate tissue-specific transcription response to BPA in addition to seasonally related changes in estrogenic activity reported previously [19,34,35].

Observed increases in CYP19a and CYP19b mRNA transcripts in fish exposed to 20 μg/L BPA are in agreement with previous works on medaka and zebrafish exposed to a high concentration of BPA [36–38]. These suggest that P450 aromatase family are targets of BPA and can be used as a biomarker for screening reproductive disruption.

The observed stimulation of Vtg mRNA transcript indicates estrogenic property of BPA at 20 μg/L in the present study, which encompasses environmental level. Stimulation of Vtg synthesis in liver has been frequently reported in response to BPA, for instance, in common carp [13], goldfish [22],

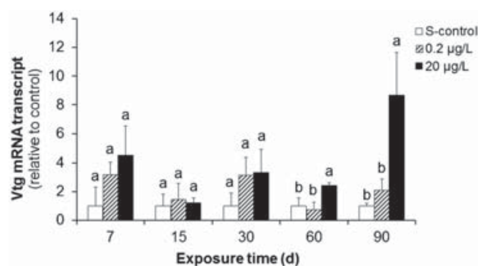

Fig. 7. Mean ± SEM messenger RNA (mRNA) transcript of vitellogenin receptor (Vtg) in the liver of male goldfish exposed to bisphenol A (BPA). The fish were exposed to nominal 0.2 and 20 μg/L BPA and transcript abundance is expressed relative to that of solvent control (0.001% dimethylsulfoxide [DMSO]). At each exposure time, values with different superscripts are significantly different ($n = 3–8$, $p < 0.05$).

swordtail [39], fathead minnow [10,40], medaka [11,12], and rainbow trout [41]. The reported effective concentrations for inducing Vtg production are different among these studies that might be addressed to BPA uptake rates, species-specific ER binding affinities, fish species, age, maturity stage, and exposure period. However, all of these observations suggest estrogenic activity of BPA at high concentration, which is similar to other estrogens such as (E_2 and 17β-ethynylestradiol) or estrogen-mimicking compounds such as nonylphenol and phthalate [40,42].

Observed decrease of sperm quality is in agreement with Lahnsteiner et al. [26] and Hatef et al. [22], when brown trout and goldfish were exposed to 1.75 to 2.40 and 0.6 to 11 μg/L BPA, respectively. Bisphenol A may modulate sperm maturation via alternations in sex steroid biosynthesis in testis [22]. In mammals, a concentration-dependent reduction in sperm motility has been reported after the administration of BPA to male rats [24,43]. Meeker et al. [44] reported a relationship between sperm motility and urinary BPA in men examined in an infertility clinic. However, the mechanisms of adverse effects of BPA on sperm quality and sperm motility are still unknown. Further studies are required to investigate modes of action of BPA on sperm maturation by studying sperm maturation–inducing hormone, intracellular cyclic adenosine monophosphate and pH in sperm, initial adenosine triphosphate content, plasma membrane potential for sperm activation [45–47], as well as sperm cytoskeletal proteins, DNA damage, and oxidative stress [30,43,44].

A model is proposed for the adverse effects of BPA on reproductive function, based on the results of the present study and previous findings (Table 5, Fig. 8). At low concentrations, BPAs decrease androgens via modulation in testicular steroidogenesis functions, particularly because of a decrease in substrate availability mediated by suppression StAR. These changes could lead to a decrease in sperm quality. At high concentrations, BPA induces Vtg production in the liver mediated by stimulation in mRNA transcript of ERs and P450 aromatase, which converts androgens to estrogen. Under physiological conditions, all three ERs contribute in Vtg [15]. However, ERα expression is induced by E_2 through activation of ERβ subtypes, and ERβ1 is responsible for maintaining the ERα level in the liver. The present study suggests that BPA induces Vtg induction, particularly mediated by ERβ subtypes, because ERα expression remained unchanged in the liver.

Table 5. Summary of the bisphenol A (BPA) effects on messenger RNA (mRNA) transcripts of estrogen and androgen receptors, vitellogenein, and steroidogenesis mediating genes in male goldfish (*Carassius auratus* L.)

		0.2 μg/L BPA		20 μg/L BPA	
Gene	Organ	Short-time exposure (7–15 d)	Long-time exposure (30–90 d)	Short-time exposure (7–15 d)	Long-time exposure (30–90 d)
StAR	Testis	NS	↓	NS	NS
AR	Testis	NS	NS	NS	↑
CYP19a	Testis	NS	NS	NS	↑
CYP19b	Brain	NS	NS	NS	↑
ERα	Brain	NS	NS	NS	↑
	Testis	NS	NS	NS	NS
	Liver	NS	NS	NS	NS
ERβ1	Brain	NS	NS	NS	↑
	Testis	NS	NS	↑	↑
	Liver	NS	↑	NS	↑
ERβ2	Brain	NS	NS	NS	↑
	Testis	NS	↑	NS	↑
	Liver	NS	NS	NS	↑
Vtg	Liver	NS	NS	NS	↑

NS = no significant effect was observed; StAR = steroidogenic acute regulatory protein; AR = androgen receptor; CYP19a = testis aromatase; CYP19b = brain aromatase; ERα, ERβ1 and ERβ2 = estrogen receptor subtypes; Vtg = vitellogenin.

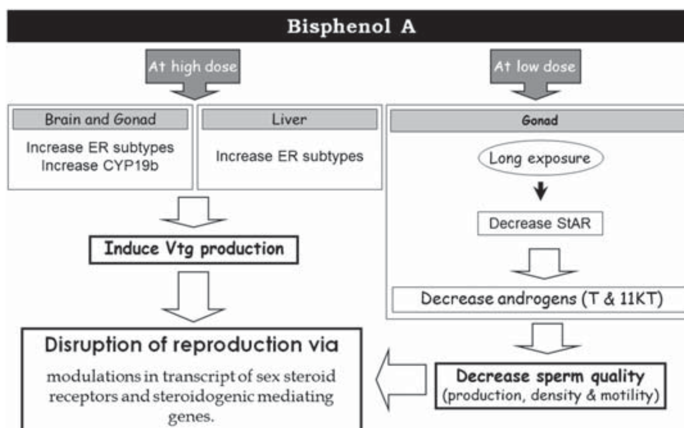

Fig. 8. A model summary for the effects of bisphenol A (BPA) on reproductive mediated genes in goldfish at low and high environmentally relevant concentration. ER = estrogen receptor; CYP19b = brain aromatase; StAR = steroidogenic acute regulatory protein.

In conclusion, the present study shows that the BPA-mediated actions likely involve a number of mechanisms, including changes in ER expression, aromatase activity, and potential pathways interfering with androgen-sensitive response. The BPA mode of action is dose-dependent, but it interferes with testicular functions and reduces the sperm quality. Further studies are needed to understand the potential roles of sex steroids and their receptors in sperm maturation or signaling cascades required for sperm activation.

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Di-(2-ethylhexyl)-phthalate IMPAIRS SPERM QUALITY IN GOLDFISH ASSOCIATED WITH DISRUPTION IN ANDROGENESIS

Azadeh Hatef [a], Sayyed Mohammad Hadi Alavi [a], Sylvain Milla [b], Ian A. E. Butts [c], Marek Rodina [a], Oliana Carnevali [d], Pascal Fontaine [b], Otomar Linhart [a]*

[a] *University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodňany 389 25, Czech Republic*
[b] *Université de Lorraine, UR. AFPA, Equipe DAC, 2 Avenue de la Forêt de Haye, Vandoeuvre-les-Nancy, F-54505, BP 172, France*
[c] *Department of Biological Sciences, University of Windsor, 401 Sunset Ave., Windsor, ON, N9B 3P4, Canada*
[d] *Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy*
[*] *Corresponding author: Tel.: +420 387 774 610, Fax: +420 387 774 634, E-mail: alavi@frov.jcu.cz*

ABSTRACT

Di-(2-ethylhexil) phthalate (DEHP) is an endocrine disrupting chemicals which has been shown to have adverse effects on the male reproductive system. In contrast to mammals, mechanisms of toxicity of DEHP are largely unknown in fish especially with regard to sperm quality as endpoint for male fertility. In this work, reproductive impairments were studied in male goldfish (*Carassius auratus*) exposed to nominal 1, 10, and 100 µg/L DEHP and compared with the effects of 17β-estradiol (E_2) (nominal 5 µg/L) to elucidate the roles of sex steroid receptors and steroidogenesis mediating genes. Exposure to DEHP reduced sperm volume at 1 µg/L, velocity at 10 µg/L and motility at 100 µg/L, while no sperm was produced in fish exposed to E_2. Both DEHP and E_2 inhibited 11-ketotestosterone synthesis, and as expected the E_2 level was increase in fish exposed to E_2. In DEHP (100 µg/L) and E_2 exposed fish, steroidogenic acute regulatory protein (StAR) and CYP1A mRNA transcripts were decreased suggesting disruption in transfer of cholesterol to sex steroidogenesis pathway in testis or its metabolism in liver, respectively. DEHP did not affect vitellogenin (Vtg) and androgen (AR) mRNA transcripts. Estrogen receptor (ERα) and P450 aromatase (CYP19b) mRNA transcripts were decreased in the liver and brain at 1 µg/L DEHP, respectively. In contrast, E_2 induced Vtg mRNA transcript associated with increase in AR, ERα and CYP19b. Our results suggest steroid receptor independent mechanism for DEHP toxicity leading to a decrease in fish sperm quality. In this context, 11-KT level and StAR and CYP1A transcriptions are recommended as DEHP indicators for environmental risk assessment.

Keywords: *11-ketotestosterone; 17β-estradiol; CYP19A; StAR; steroid receptors; vitellogenin*

1. INTRODUCTION

Phthalate esters are widely used plasticizers, which are not covalently bound to polymeric matrix and can leak out into the surrounding environment (Kastner et al., 2012). They can be found at measurable concentrations in aquatic ecosystems and are universally considered as an endocrine disrupting chemicals (EDCs) (Staples et al., 1997; Bauer and Herrmann, 1997). Several studies have shown adverse

reproductive effects in mammals or fish exposed to most phthalates, which lead to decrease in sperm or egg quality and fertility (Foster et al., 2001; Latini et al., 2006; Howdeshell et al., 2007; Carnevali et al., 2010; Uren-Webster et al., 2010; Gentry et al., 2011).

Di-(2-ethylhexil) phthalate (DEHP) is among the most used plasticizers in the industry with an annual production of 260 million pounds, which represent a quarter of all phthalates produced and widespread in the aquatic environment (EU, 2008). The environmental concentration is normally between 2 and 3 μg/L and has been also measured up to 98-219 μg/L in "hotspots" (Fromme et al., 2002). The Environmental Protection Agency has established a DEHP safety concentration limit in drinking water to be 6 μg/L (Clark et al., 2003). Moreover, DEHP has been found in fish tissue (for instance, 1-2.6 mg/kg in fish from Austrian rivers) (European Commission, 2003).

Preliminary studies hypothesized that most phthalates (such as butyl benzyl phthalate (BBP), dibutyl phthalate (DBP), diisobutyl phthalate (DiBP)) act as estrogenic chemicals due to their affinity to bind to estrogen receptors (ER) with potencies approximately $0.5–1 \times 10^6$ times less than 17β-estradiol (E_2), but DEHP showed no estrogenic activity, *in vitro* (Harries et al., 1997). In contrast, Takeuchi et al. (2005) have reported estrogenic, anti-estrogenic and anti-androgenic activities of most phthalates and concluded that several phthalate esters simultaneously act as agonists and/or antagonists via one or more hormonal receptors. In this context, DEHP exhibited both estrogenic and anti-estrogenic activity without affinity to androgen receptor (AR) (Takeuchi et al., 2005). Recent studies showed potential anti-androgenic effects of DEHP via alterations in expression of genes encoding enzymes involved in androgenesis in mammals, which has led to decrease in testosterone (T) (Corton and Lapinskas, 2005; Foster, 2006; Swan, 2008).

Compared to mammals, there is a significant lack of research investigating the effects of phthalates including DEHP on male reproductive health in fish, particularly about sex steroid synthesis, sperm dysfunctions and transcriptomic alterations of steroid receptors and steroidogenesis mediating genes at environmentally relevant concentrations. Harries et al. (2000) reported no adverse effects of BBP on reproduction in fathead minnows following 3 weeks exposure to 71 μg/L BBP. While decrease in fertility has been reported in male zebrafish injected with 5,000 mg/kg DEHP following 6–10 days (Uren-Webster et al., 2010). Disruption in spermatogenesis has been reported in zebrafish injected with 5,000 mg/kg DEHP or in fathead minnow exposed to 12 μg/L DEHP. These effects have been associated with increase in peroxisome proliferation signaling in the testis and oestrogen signaling in the liver (Uren-Webster et al., 2010) or via alternations in steroid metabolism, but not peroxisome proliferation (Crago and Klaper, 2012). DEHP has been shown to reduce activity of genes involved in testicular androgenesis (Thibaut and Porte, 2004). However, none of previous studies showed inhibition of androgen synthesis (either T or 11-ketotestosteron, 11-KT) (Thibaut and Porte, 2004; Uren-Webster et al., 2010; Crago and Klaper, 2012).

Taken together, literature on DEHP effects on reproductive health is rare and somewhat contradictory and required more examination to establish the mechanisms of its toxicity. Therefore, in this study the effects of exposure to a range of concentrations of DEHP were investigated on the reproductive physiology in male goldfish (Carassius auratus) following one month exposure during spawning season. The goldfish was used, because several key genes involved in reproduction have been previously cloned and characterized and because our previous studies showed sensitivity of goldfish to endocrine disrupting chemicals (EDCs) (Choi and Habibi, 2003; Hatef et al., 2012a, b, c). Firstly we investigated whether DEHP causes any reproductive disruption at environmentally relevant concentration by quantifying sperm quality and sex steroid levels. In this regard, sperm volume, motility and velocity were evaluated as well as 11-KT (major androgen in fish) and E_2. The second aim was to identify reproductive biomarkers representing DEHP effects. In this context, transcript profiles for genes linked to sex steroid were evaluated in order to develop a screening system for ecotoxicity monitoring and

to elucidate the pathways of reproductive disruption mediating by DEHP in fish. Since DEHP effects via oestrogen pathways has been previously suggested (Thibaut and Porte, 2004; Uren-Webster et al., 2010), a group of fish was exposed to E_2, an estrogenic compound, which induces vitellogenin (Vtg) induction in liver via ER mediated pathway (Nelson and Habibi, 2010). The E_2 positive control provides valuable information for a more comprehensive understanding of the potential threat of DEHP on the reproductive status of fish.

2. MATERIAL AND METHODS

2.1. Experimental design and sampling

Mature male goldfish (2–3 years-old) were firstly checked for their health and acclimatized for two weeks before introduction into the aquaria (each 80 L). All fish were kept under similar temperatures and 12 h light /12 h dark photoperiod. Water temperature was measured 18 and 22 °C at the beginning and end of experiment, respectively. Dissolved oxygen and pH of the water were 6.0 ± 0.5 mg/L and 7.5 ± 0.3, respectively. The fish were fed once a day (3% body weight) with commercial food (ZZN Vodnany, Czech Republic). All animals were handled according to §17 odst. 1 zakona No. 246/119 Sb, Ministry of Agriculture of the Czech Republic, approved by Central Ethics Committee of the Ministry of Health of the Czech Republic; all efforts were made to minimize suffering.

Fish were exposed to DEHP at nominal 1, 10 and 100 µg/L dissolved in acetone (solvent control) and E_2 (nominal 5 µg/L) as an estrogenic control. A control group without acetone was also studied, but results did not show any significant difference with the solvent control (data are not shown). Therefore, only the data for the solvent control containing acetone were used as reference for comparison with respective treatments. Every 48 h, 80% of the exposure solution was renewed. Thirty days following treatment, goldfish were anaesthetized with 2-phenoxyethanol dissolved in water (0.3 mL/L) before sampling. Body mass (± 0.1 g) and total length (± 1 mm) for six males from each treatment were measured. Then, sperm and blood samples were collected according to Hatef et al. (2012a). Immediately fish were sacrificed and their brain, testis and liver were removed. Gonadosomatic index (GSI = gonad weight / body weight × 100) and hepatosomatic index (HSI = liver weight / body weight × 100) were evaluated. The tissues were carefully dissected and immediately frozen in liquid nitrogen and stored at -80 °C until further analysis.

2.2. Sperm quality assessment

Sperm volume, motility and velocity were measured for monitoring sperm quality. To evaluate motility and velocity, sperm of each individual was directly activated in NaCl 50 mM, KCl 5 mM, Tris 20 mM, pH 8.5 at ratio 1:1000–2000. Bovine serum albumin (0.1% w/v) was added into the activation medium to prevent sperm from sticking to the slides. Sperm motility was recorded using a CCD video camera (SONY DXC-970MD, Japan) mounted on a dark-field microscope (Olympus BX50, Japan) equipped with stroboscopic lamp. Then, the successive positions of sperm heads were captured using a DVD-recorder (SONY DVO-1000 MD, Japan) and analyzed with a micro image analyzer (Olympus Micro Image 4.0.1. for Windows) (for details see Hatef et al., 2012b). Sperm motility and velocity of only motile cells were measured from two separate records for each individual and the mean of these values were used in statistical analysis.

Table 1. *Primer sequences and amplification (Annealing) programs used for measurements of selected genes using qRT-PCR.*

Gene	Forward Primer	Reverse Primer	Annealing temperature (°C)
StAR	ATGGCTGGCAAACTGAGATCGAGA	TCCATGTTATCCACCAGCTCCTCA	57
CYP19a	TTGTGCGGGTTTGGATCAATGGTG	TTCCGATACACTGCAGACCCAGTT	55
CYP19b	AGGCGAGCGGGATGTAGAGT	CGTCCGATGTTCAGGATGAGG	58
Vtg	GAAGTGCGCATGGTGGCTTGTATT	AGCTGCCATATCAGGAGCAGTGAT	55
ERα	GAGGAAGAGTAGCAGCACTG	GGCTGTGTTTCTGTCGTGAG	55
AR	GATGAAAGGTCCAGAGGAGG	ACTGTGAGTGGAACGTCAGG	55
GAPDH	TGATGCTGGTGCCCTGTATGTAGT	TGTCCTGGTTGACTCCCATCACAA	57

StAR, steroidogenic acute regulatory protein; CYP19a, testis aromatase; CYP19b, brain aromatase; Vtg, vitellogenin; ERa, estrogen receptor subtype; AR, androgen receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

2.3. Sex steroids measurement

The E_2 was assayed on 50 µL of plasma diluted from 1/3 to 1/10 in a specific buffer, using the Diasource EIA kit (Diasource, Nivelles, Belgium). Fifty µL of each E_2 standard, control and plasma samples were dispensed in each well of a 96 well plate. Fifty µL of E_2-horseradish peroxidase conjugate were added into each well as well as 50 µL of antibody directed against E_2. 200 µL of substrate solution were added after 120 min of incubation at room temperature, and 4 times washing. The plate was incubated for 30 min at room temperature and the reaction was stopped by adding 100 µL of H_2SO_4 (1.8 N). The optical density was read at 450 nm with a microtiter plate reader. Sensitivity was 5 pg/ml, and intra-assay and inter-assay coefficients of variation were respectively 4% and 6% for a plasma concentration in the range of 100–250 pg/mL. Concerning 11-KT, the Cayman EIA kit was used (Cayman, Michigan, USA) using plasma diluted 10 times. Similarly to E_2 assay, fifty µL of plasma/standards, tracer and antibody were added into each well. After 120 min of incubation and 5 times washing, 200 µL of substrate solution were added. This step was followed by a 90 min incubation step before reading the absorbance at 405 nm. Sensitivity was 1.3 pg/mL and intra-assay and inter-assay coefficients of variation were respectively 8% and 9% for a plasma concentration of 6 pg/mL.

2.4. RNA extraction, cDNA synthesis and real-time PCR

For each individual, total RNA was extracted from the liver, testis and brain using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 40 mg of tissue were homogenized in 1 mL of Trizol and 200 mL of chloroform was added. After mixing, samples were centrifuged (12,000 g for 15 min). Supernatant was transferred in a new tube containing an equal volume of isopropanol. Mixture was centrifuged (12,000 g for 10 min) and the precipitated RNA pellet was washed once using 1 mL of ethanol 75%. Total RNA was finally dissolved in diethyl pyrocarbonate water. A NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmigton, USA) was used to measure RNA concentration (absorbance at 260 nm) and purity assessed from the 260 nm/280 nm absorbance ratio. Samples were stored at -80° C until further use.

One µg of RNA was used for cDNA synthesis, employing iScript cDNA Synthesis Kit (Bio-Rad). cDNA was diluted at 1:10 before qRT-PCR using an iQ5 iCycler thermal cycler (Bio-Rad Laboratories inc, Milano, Italy) with SYBR Green. The reactions (diluted cDNA 1 µL, 5 µL 2× concentrated iQ SYBR Green Supermix

containing SYBR Green as a fluorescent intercalating agent, forward and reverse primers each 0.3 µL and RNase free DW 3.4 µL) were set on a 96-well plate. Table 1 shows the primer sequences used for each gene. Primer efficiencies determined on serial dilutions of cDNA were all between 90% and 100%. For each sample, qRT-PCR was run in duplicate or triplicate to ensure consistency. The thermal profile for all reactions was 3 min at 95 °C and 45 cycles of 30 s at 95 °C, 60 °C and 72 °C. Fluorescence monitoring occurred at the end of each cycle. Relative expression levels were determined by normalizing to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene. No amplification product was observed in non-template controls and no primer-dimer formations were observed in the control templates. Results were determined using method of Livak and Schmittgen (2001).

2.5. Statistical analysis

Data were analysed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, U.S.A.). Residuals were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). As needed, data were transformed to meet assumptions of normality, and homoscedasticity. One-way ANOVA followed by a Tukey-Kramer test were used to analyse the effects of treatments (DEHP and E_2) on each parameter. All data are presented as mean ± standard error of mean (S.E.M.).

3. RESULTS

3.1. Fish body mass, total length, GSI and HSI

No mortality was observed in any treatments during the period of experiment. No significant differences in body mass, total length or HSI were found ($p > 0.05$, Table 2). GSI in E_2 group was lower than that of fish in both DEHP and control groups ($p < 0.001$,Table 2).

3.2. Sperm volume, motility and velocity

No sperm was produced in fish exposed to E_2. Sperm motility evaluated at 15 s post activation showed decrease in fish exposed to 100 µg/L DEHP ($p < 0.01$, Fig. 1a), while sperm velocity was lower at 10 and 100 µg/L DEHP ($p < 0.001$, Fig. 1b). Sperm volume was decreased in all DEHP groups compared to control ($p < 0.01$, Fig. 1c).

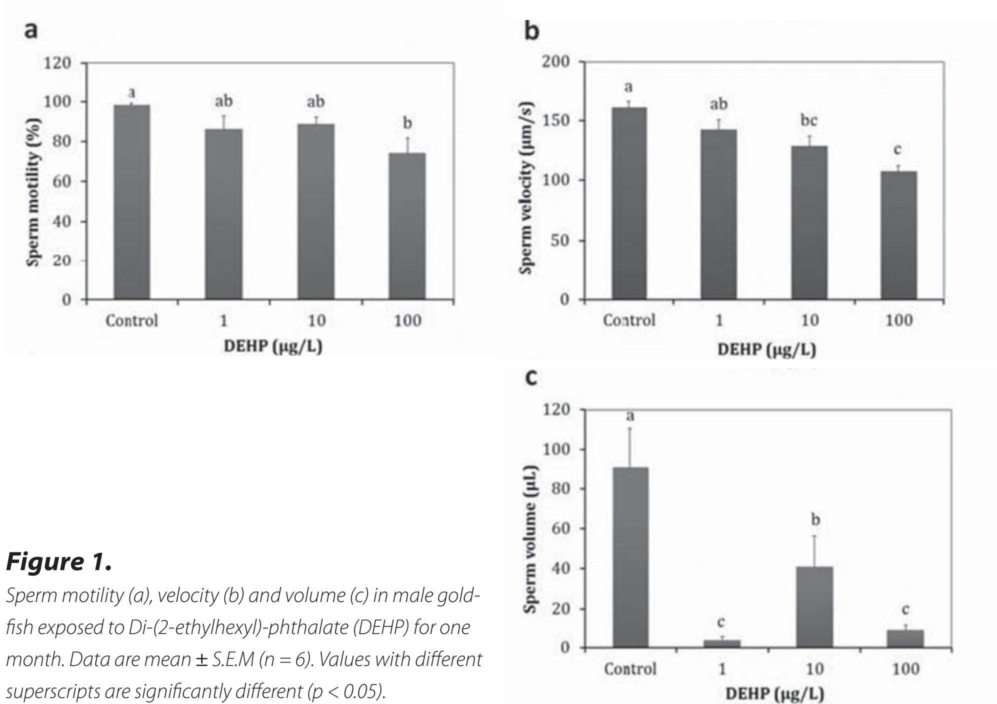


Figure 1.

Sperm motility (a), velocity (b) and volume (c) in male goldfish exposed to Di-(2-ethylhexyl)-phthalate (DEHP) for one month. Data are mean ± S.E.M (n = 6). Values with different superscripts are significantly different (p < 0.05).

3.3. Sex steroids (11-KT and E_2)

E_2 level in blood plasma was unchanged in DEHP groups, but as expected it was increased in fish exposed to E_2 ($p < 0.001$, Fig 2a). Decrease in 11-KT level in the blood plasma was observed in both DEHP and E_2 group compared to control ($p < 0.001$, Fig. 2b).

3.4. Transcriptomic alterations in brain, testis and liver

In the brain, the transcripts of CYP19b and StAR were measured. Transcript of StAR mRNA was only decreased in fish exposed to E_2 ($p < 0.001$), but unchanged across DEHP treated groups ($p > 0.05$) (Fig. 3a). CYP19b mRNA transcript was decreased in fish exposed to 1 µg/L DEHP, while E_2 induced its mRNA level compared to control ($p < 0.001$, Fig. 3b).

Table 1. *Body mass (g), total length (cm), gonadosomatic index (GSI, %) and hepatosomatic index (HSI, %) of male goldfish exposed to Di-(2-ethylhexyl)-phthalate (DEHP) and 17β-estradiol (5 μg/L) for one month. Data are mean ± S.E.M (n = 6).*

DEHP (μg/L)	Total length	Body mass	GSI	HSI
control	12.7 ± 0.5a	27.5 ± 3.5a	3.7 ± 0.9a	3.3 ± 0.2a
1	12.6 ± 0.2a	25.7 ± 1.5a	2.3 ± 0.4a	3.2 ± 0.3a
10	12.2 ± 0.2a	27.4 ± 1.3a	3.8 ± 0.3a	3.1 ± 0.2a
100	12.5 ± 0.2a	24.5 ± 2.2a	3.9 ± 0.6a	2.7 ± 0.2a
17β-estradiol	12.0 ± 0.5a	24.6 ± 2.4a	0.7 ± 0.3b	2.9 ± 0.3a

For each parameter, values with different superscripts are significantly different (p < 0.05).

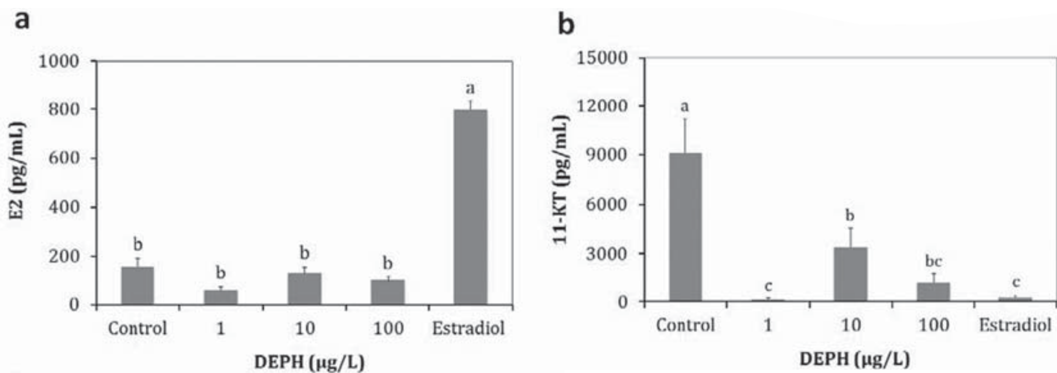


Figure 2. *17β-estradiol (E_2, a) and 11-ketotestosterone (11-KT, b) levels in blood plasma of goldfish exposed to Di-(2-ethylhexyl)-phthalate (DEHP) and 17β-estradiol (5 μg/L) for one month. Data are mean ± S.E.M (n = 6). Values with different superscripts are significantly different (p < 0.05).*

In the testis, the transcripts of StAR, CYP19a and AR were quantified. StAR mRNA transcript was decreased in DEHP and E_2 groups compared to control ($p < 0.001$, Fig. 4a). Transcripts of CYP19a and AR mRNA were unchanged ($p > 0.05$, Fig. 4b, c, d).

In the liver, the transcripts of Vtg, ERα, AR and CYP1A were quantified. Transcript of Vtg (Fig. 5a) and AR (Fig. 5c) mRNA were unchanged in DEHP treated groups. ERα mRNA was decreased in fish exposed to 1 μg/L DEHP ($p < 0.01$, Fig. 5a). Increase in Vtg (Fig. 5a), ERα (Fig. 5b) and AR (Fig. 5c) mRNA transcripts were observed in fish exposed to E_2 ($p < 0.001$). CYP1A mRNA transcript was decreased in fish exposed to 100 μg/L DEHP; the results were comparable to that obtained with E_2 exposure ($p < 0.05$, Fig. 5d).

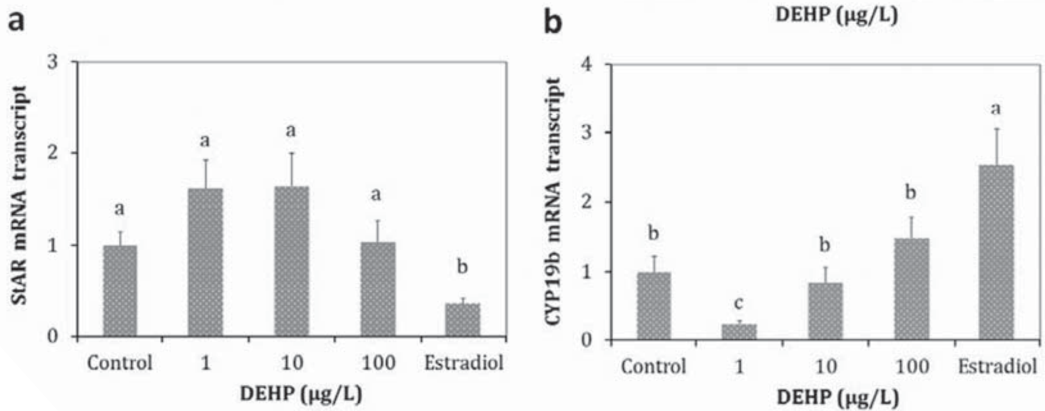
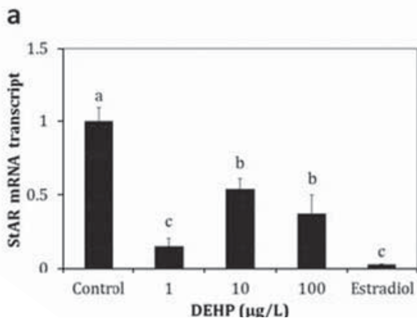
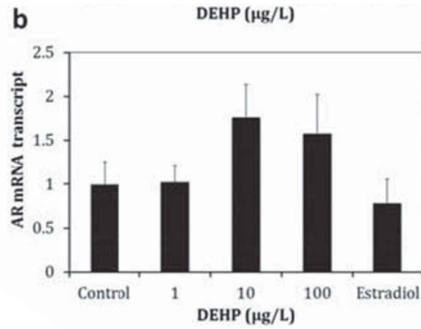


Figure 3. *Transcript abundance of steroidogenic acute regulatory protein (StAR, a) and P450 aromatase (CYP19b, b) in brain of male goldfish exposed to Di-(2-ethylhexyl)-phthalate (DEHP) and 17β-estradiol (5 μg/L) for one month. Data are mean ± S.E.M (n = 6). Values with different superscripts are significantly different ($p < 0.05$).*

4. DISCUSSION

Our data shows DEHP as a potent disruptor of testicular androgenesis which is associated with decrease in StAR and CYP1A mRNA transcript leading to decrease in sperm production, motility and velocity as critical points for male fertility in fish. To our knowledge, reduction in 11-KT level resulted from disruption in transfer of cholesterol to sex steroidogenesis pathway and subsequent influence on sperm quality have not been previously reported as well as decrease in StAR and CYP1A mRNA transcripts. These results clearly demonstrate anti-androgenic activity of DEHP at environmentally relevant concentration. Moreover, the present study shows DEHP functional similarities to other anti-androgens such as cyproterone acetate, bisphenol A and vinclozolin, which influence reproduction via decrease in 11-KT synthesis (Sharpe et al., 2004; Hatef et al., 2012a, b). However, mechanisms of toxicity may differ, because our data suggest DEHP action through sex steroid receptor independent pathway while the other anti-androgens acts through competitive binding to AR thus inhibiting androgen synthesis in testis.





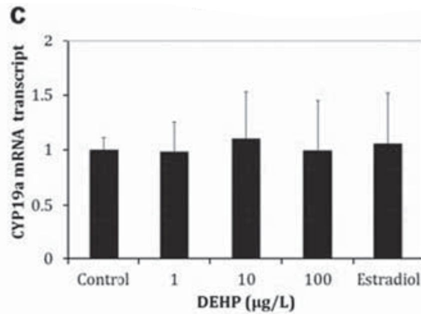
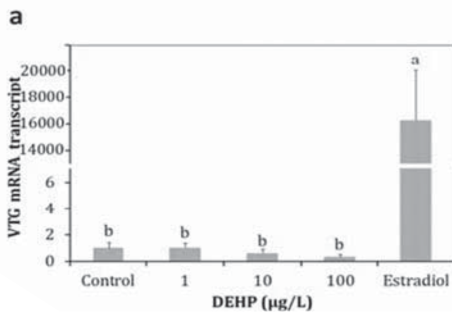
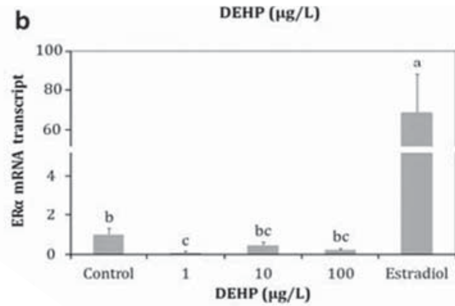
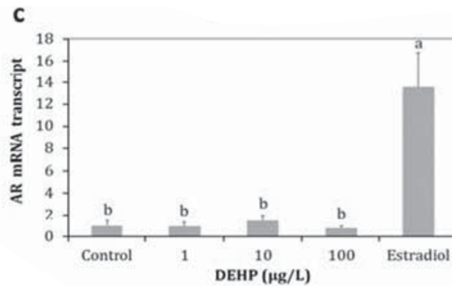


Figure 4.

Transcript abundance of steroidogenic acute regulatory protein (StAR, a), androgen receptor (AR, b) and P450 aromatase (CYP19a, c) and in testis of male goldfish exposed to Di-(2-ethylhexyl)-phthalate (DEHP) and 17β-estradiol (5 µg/L) for one month. Data are mean ± S.E.M (n = 6). Values with different superscripts are significantly different (p < 0.05).







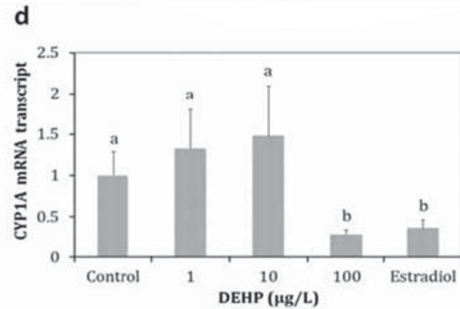


Figure 5. *Transcript abundance of vitellogenin (Vtg, a), estrogen receptor alpha (ERa, b), androgen receptor (AR, c) and P450 aromatase (CYP1A, d) in liver of male goldfish exposed to Di-(2-ethylhexyl)-phthalate (DEHP) and 17β-estradiol (5 µg/L) for one month. Data are mean ± S.E.M (n = 6). Values with different superscripts are significantly different (p < 0.05).*

4.1. GSI and HSI

Unchanged GSI in DEHP treated fish compared to the control confirms previous results in fathead minnow (Crago and Klaper, 2012) or mammals (Howdeshell et al., 2007; Pocar et al., 2012). In contrast, E_2 reduced the GSI (Kang et al., 2002; Sharpe et al., 2007, present study). These results suggest that GSI may not be the main endpoint for toxicity of DEHP in goldfish. Similar to Kang et al. (2002), HSI remained unchanged in fish exposed to DEHP or E_2. However, there is one study in male zebrafish that shows HSI increase after injection with 5,000 µg/kg DEHP (Uren-Webster et al., 2010). The increase in HSI might be addressed to very high dose of DEHP applied by these authors as it remained unchanged at lower doses that have been examined.

4.2. Sperm volume, motility and velocity

Evaluation of sperm quality is a critical endpoint of EDCs, because sperm fertility in fish is highly dependant on sperm volume, motility and velocity (Lahnsteiner et al., 2006; Linhart et al., 2008). Decrease in sperm volume, motility and velocity in fish when exposed to DEHP at environmentally relevant concentrations consistent with the mammalian observations (Lee et al., 2009; Vo et al., 2009; Pocar et al., 2012). In addition, our results provide a mechanism (i.e. reduction in male sperm quality) to explain the observed decrease in sperm fertility for zebrafish that were exposed to DEHP (Uren-Webster et al., 2010).

In the present study, males exposed to E_2 did not produce any sperm. Lahnsteiner et al. (2006) reported that E_2 reduces sperm volume at ≥1 ng/L. Sperm volume is determined by seminal plasma secretion and sperm release from testis into the spermatic duct. These phenomena are regulated by major androgen in fish (11-KT) (Borg, 1994; Young et al., 1995). Therefore, observed decreases in 11-KT level suggest the hypothesis that both DEHP and E_2 act on testis causing a reduction in sperm production by 11-KT biosynthesis disruption, which eventually leads to partial or absolute inhibition of spermatogenesis.

Adverse effects of DEHP on sperm motility suggest disruption in spermatozoa maturation, which is an important step for acquisition of potential for sperm activation (Miura et al., 1992) or damage to sperm cells (MacAllister and Kime, 2003; Hatef et al., 2012b). Sperm velocity also depends on flagellar beating, which is ATP-dependent activity (Alavi et al., 2009). Decrease of sperm velocity in fish exposed to DEHP (present study) or E_2 (Lahnsteiner et al., 2006) might be contributed to ATP content and regeneration of ATP (Butts et al., 2010); suggesting that mitochondria may be a potential site of phthalates (Oishi, 1990). Lahnsteiner et al. (2006) reported that sperm motility and fertility decreased in salmonids exposed to 1 ng/L E_2.

4.3. Sex steroids (11-KT and E_2)

In the present study, we evaluated 11-KT, because it is major androgen in fish, which regulates spermatogenesis and involves in sperm maturation (Borg, 1994; Young et al., 2005). Observed DEHP effect on 11-KT decrease in fish is novel and consistent with previous studies that have shown similar reduction in mammalian androgen, i.e., T (Culty et al., 2008; Lee et al., 2009; Hannas et al., 2011). There is one study that shows no T change in fathead minnow exposed to 12 µg/L DEHP, but decrease was reported after exposure to mixture of DEHP (12 µg/L) and another anti-androgen, herbicide linuron (1 µg/L) (Crago and Klaper, 2012). One study also shows increase in 11-KT level of common carp exposed to a very high dose of 20×10^3 µg/L DEHP (Han et al., 2009). The contradictory results of DEHP might be related to the different concentrations employed. In E_2 exposed fish, 11-KT was decreased which

is consistent with other studies in fish or mammals (MacLatchy et al., 1997; Sharpe et al., 2007; Scott et al., 2009; Hannas et al., 2011). E_2 concentration remained unchanged in goldfish exposed to DEHP indicating that DEHP does not deeply alter estrogen production. This is in contrast to previous study on fathead minnow exposed to 12 μg/L DEHP, which resulted in decrease of E_2. The authors concluded anti-estrogenic effect of DEHP (Crago and Klaper, 2012). Increase in E_2 synthesis in fish exposed to E_2 confirms estrogenic activity of E_2 (Nelson and Habibi, 2010).

4.4. Transcriptomic alterations in brain, liver and testis

Steroidogenic regulation shares similarities across vertebrates and involves a series of steroidogenic enzymes (Payne and Hales, 2004). We studied transcript level of sex steroid receptors and of steroidogenic enzymes in different reproductive organs (brain, testis and liver) to understand the mechanisms underlying DEHP effects. Previous studies have focused on testis (Crago and Klaper, 2012) or testis and liver (Uren-Webster et al., 2010).

This study shows StAR mRNA transcript decrease in the testis of both DEHP and E_2 exposed fish, which is consistent with previous studies on mammals (Howdeshell et al., 2007; Vo et al., 2009; Culty et al., 2008). In fish, previous study showed no alternation in testicular StAR mRNA in fathead minnow (Crago and Klaper, 2012). The present results suggest disruption in the transfer of cholesterol to steroidogenesis pathway as a critical point of DEHP anti-androgen effect, which decrease precursor for 11-KT synthesis. Moreover, our data shows that both DEHP and E_2 exhibit similar action to decrease StAR mRNA level leading to inhibition of androgenesis. In brain of fish exposed to DEHP, StAR mRNA transcript was unchanged, but decreased in E_2 exposed fish. Therefore, StAR transcript alterations observed in the brain of E_2 exposed fish suggests potential neuroendocrine role of StAR in androgenesis in addition to testicular StAR.

To study possible anti-estrogenic activity of DEHP suggested by Crago and Klaper (2012), transcript alterations in aromatase P450 were studied in the brain (CYP19b) and testis (CYP19a) and compared with E_2. Among them CYP19b seems to be estrogen-responsive elements in fish that converts androgens into estrogens (Callard et al., 2001). This study showed no change in CYP19a mRNA transcript, which is in agreement with previous study on fathead minnow males (Crago and Klaper, 2012). This is different from mammals, which decrease in CYP19 has been frequently reported in testis after exposure to DEHP (Lee et al., 2009; Vo et al., 2009; Pocar et al., 2012). In the present study, CYP19b was decreased in goldfish exposed to 1 μg/L DEHP which may explain the observed trend toward decreased E_2 in goldfish exposed to the same concentration. However, our data is not well enough to support previous anti-estrogenic activity of DEHP hypothesized by Crago and Klaper (2012), because neither E_2 nor ER was significantly changed following exposure of goldfish to DEHP. Our data shows an increase of CYP19b in fish exposed to E_2, but CYP19a remain unchanged. These results are in agreement with previous studies in which exposure to estrogenic compounds caused up regulation of CYP19 in brain, but failed to modulate CYP19 in the testis (Kishida et al., 2001; Kazeto et al., 2004; Guyon et al., 2012).

CYP1A is a detoxifying gene transcribed spatially in the liver of fish (Olsvik et al., 2007). It is a pollutant-metabolizing enzyme and its suppression attribute to high E_2 level in plasma (Elskus, 2004). Our study showed significant decrease of CYP1A mRNA transcript in the liver of fish exposed to 100 μg/L DEHP and E_2, which confirm previous results showing inhibitory function of E_2 on CYP1A enzyme activity (Elskus, 2004; Hasselberg et al., 2004). Decrease of CYP1A mRNA transcript in the liver suggests that both DEHP and E_2 may act by suppressing metabolizing activity in the liver strengthening their toxic effects.

We analyzed the transcript profiles of ERα, AR and Vtg involved in estrogen and androgen signaling pathways to search for their involvement in the response of male goldfish to DEHP. In fish exposed to

DEHP, our data showed no difference in the relative mRNA transcript levels of AR and ERα in the brain, testis and liver, except for ERα decreased in fish exposed to 1 μg/L DEHP in liver. These data are consistent with other studies examining DEHP effects in other fish species (Uren-Webster et al., 2010; Crago and Klaper, 2012) and suggest that the DEHP mode of action do not modify sex steroid receptivity. Vtg mRNA transcript levels also remained unchanged in the liver. There is one study that shows estrogenic activity of DEHP via ER mediated pathways in the liver at high concentrations (in zebrafish injected with 5000 mg/kg/bw DEHP) (Uren-Webster et al., 2010). In contrast to DEHP, this study shows effects of E_2 via alterations in ERα, AR and Vtg mRNA transcript. Similar to mammals (Thibaut and Porte 2004; Scott et al., 2009), increase in ERα, AR and Vtg mRNA in our study are consistent with previous studies in fish and suggest estrogenic effect of E_2 mediated by alterations in sex steroid, their receptors and Vtg transcripts (Christiansen et al., 2000; Nelson and Habibi, 2010). In this study, we only analyzed ERα subtype, but previous studies show no change in ERβ subtypes in fish exposed to DEHP (Uren-Webster et al., 2010; Crago and Klaper, 2012).

In conclusion, the present study characterizes the potency of DEHP for disrupting reproductive function in goldfish and compares with adverse effects of E_2, a well-known estrogenic EDC. Our data shows that both DEHP and E_2 cause adverse effects on sperm quality resulted from disruption of testicular steroidogenesis. Both DEHP and E_2 inhibit 11-KT production, while E_2 production was only induced in fish exposed to E_2. Anti-androgenic activity of DEHP might be related to transfer of cholesterol to steroidogenesis pathway or its metabolism in the liver, because StAR and CYP1A mRNA transcript decreased, respectively. Anti-androgenic activity of DEHP could not be through competitive binding to AR. Unchanged ERα and Vtg mRNA transcript reveals non-estrogenic activity of DEHP. Mechanisms of action of DEHP differ from that of E_2. The E_2 estrogenic activity is mediated by CYP19b which regulates conversion of androgens into estrogens and induces Vtg mRNA transcript via ER-mediated pathway. Similar to DEHP, E_2 toxicity might be also related to CYP1a expression in the liver. This study suggests different biomarkers-indicators for DEHP and E_2 within the context of screening for environmental risk assessment. Sex steroid levels and genes encoding enzymes for steroidogenesis (StAR for both DEHP and E_2 and CYP19a for E_2) are recommended to address this objective. However, sex steroid receptors and Vtg mRNA transcript can be only considered for E_2 toxicity.

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CHAPTER 5

GENERAL DISCUSSION ◆ ENGLISH SUMMARY ◆ CZECH SUMMARY ◆ ACKNOWLEDGEMENTS ◆ LIST OF PUBLICATIONS ◆ TRAINING AND SUPERVISION PLAN DURING STUDY ◆ CURRICULUM VITAE

GENERAL DISCUSSION

Series of *in vitro* and *in vivo* studies show the effects of endocrine disrupting chemicals (EDCs) including mercury chloride ($HgCl_2$), Bisphenol A (BPA) and Di-(2-ethylhexyl)-phthalate (DEHP) and vinclozolin (VZ) on male reproductive physiology and sperm function in fish. Using *in vitro* tests, results showed no effects of EDCs at environmentally relevant concentration. However, EDCs toxicity occurs through disruption of sperm morphology (damage to plasma membrane and flagellum) and mitochondrial functions (decrease of ATP content). In contrast, *in vivo* showed significant effects of EDCs on reproductive performances in fish, particularly sperm production, motility and velocity. Further analyses were performed to investigate alternations in testicular steroidogenesis and transcripts of sex steroid receptors, vitellogenin and sex steroid mediating genes for better understanding the EDCs modes of action.

ENDOCRINE DISRUPTING CHEMICALS AND SPERM FUNCTIONS, *IN VITRO*

Instant effects of $HgCl_2$ showed that sperm motility and velocity were reduced after activation at 34×10^3 µg/L (125 mM) $HgCl_2$, *in vitro* (Hatef et al., 2011). Similarly, previous studies showed direct adverse effects of $HgCl_2$ on sperm activation at concentrations higher than environmentally relevant concentrations. Sperm motility or velocity in African catfish (Rurangwa et al., 1998), brown trout, (Lahnsteiner et al., 2004), goldfish (Van Look and Kime, 2003), and rainbow trout (Dietrich et al., 2010) reduced when sperm was directly activated at 0.001, 0.5, 1–10, and $1–10 \times 10^3$ µg/L $HgCl_2$, respectively. Abascal et al. (2007) observed no effects of $HgCl_2$ on sperm motility in sea bass when it was added to the activation medium at concentrations up to 100×10^3 µg/L. Further experiment using demembranated sperm revealed that he observed reduction in sperm motility or velocity after instant exposure to $HgCl_2$ might be related to damage of sperm cells (Hatef et al., 2011). The ATP level in sperm cells were unchanged after direct activation in $HgCl_2$, which suggests the disruption in sperm activation does not correspond to ATP level (Hatef et al., 2011). Further experiments were performed to study effects of $HgCl_2$ on sperm functions during incubation period, because of $HgCl_2$ bioaccumulation in fish body, which has been reported up to 100 to 10,000 folds higher than its concentrations in their surrounding environment (Van Look and Kime, 2003). Following 3-24 h incubation of sperm in 85 and 850 µg/L $HgCl_2$ sperm motility and velocity were decreased which was associated with decrease in ATP contents of sperm cells as well as strong damage to sperm plasma membrane, mitochondria and the flagellar structure (Hatef et al., 2011). This results consistent with previous study on goldfish where decrease in sperm motility, velocity and flagellar length were observed after incubation of sperm at 1–10 µg/L $HgCl_2$ for 24 h (Van Look and Kime, 2003).

In vitro instant exposure of sperm lead to decrease in sperm motility and velocity at 228×10^3 µg/L (1 mM) BPA (Hatef et al., 2010), but VZ showed no effect on sperm motility at concentrations up to 20×10^3 µg/L (Hatef et al., unpublished data). Previously, effects of organic EDCs have been studied (Lahnsteiner et al., 2004; Thomas and Doughty, 2004). It was shown that 3,4-dichlorophenol at $0.1–100 \times 10^3$ µg/L and Cyclohexane at $10–100 \times 10^3$ µg/L reduces sperm motility or velocity in different fish species (Lahnsteiner et al., 2004). Thomas and Doughty (2004) observed that both non-estrogenic and estrogenic organic EDCs could interfere with a rapid nongenomic progestin action to up regulate sperm motility in Atlantic croaker. Most of these chemicals (such as BPA, nonyphenol, o,p′-DDT, p,p′-DDT, naphthalane, zearalenone or nonylphenol) partially or completely blocked up regulation of sperm motility by 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) at 0.1–10 µM, but none of them caused a decrease in percentage of motile sperm below control levels. These data indicate that organic

EDCs disrupt sperm activation at concentration, which is usually higher than environmentally relevant concentration. However, the organic EDCs can interfere with steroid, particularly progestin, receptors on sperm cells and inhibit hormonal-dependent sperm activation.

ENDOCRINE DISRUPTING CHEMICALS AND REPRODUCTIVE PERFORMANCES, *IN VIVO*

In this study, no mortality was observed when fish were exosed to BPA, DEHP or VZ, indicating that the examined concentrations of these chemicals were sublethal.

GONADOSOMATIC INDEX (GSI)

The present study showed no alternations in GSI of fish exposed to BPA (0.6–11 or 0.2–20 µg/L), VZ (100–800 µg/L) and DEHP (1–100 µg/L) (Hatef et al., 2012a, b, c, d). Similar results have been reported in fish or mammals exposed to BPA (Kang et al., 2002; Ishibashi et al., 2005; Peknicova et al., 2002; Akingbemi et al., 2004), to DEHP (Howdeshell et al., 2007; Gentry et al., 2011; Crago and Klaper, 2012) or to VZ (Makynen et al., 2000; Eustache et al., 2009; Quignot et al., 2012). However, the effect of EDCs on GSI might be related to maturity stage of examined animal. For instance, Elzeinova et al. (2008) reported no change and decrease in mice GSI at sexual differentiation and maturation stage, respectively. In contrast, the present study confirms higher potency of E_2 to alter GSI (Hatef et al., 2012b, c), which has been previously shown by Kang et al. (2002), Seki et al. (2006) and Sharpe et al. (2007).

SPERM PRODUCTION

Sperm production indices, including sperm volume, density, or total number of spermatozoa were reduced in fish exposed to 0.2–20 µg/L BPA (Hatef et al., 2012d), 1–100 µg/L DEHP (Hatef et al., 2012b) and 800 µg/L VZ (Hatef et al., 2012c). Similar reduction in sperm production was observed in fish or mammals exposed to BPA (Lahnsteiner et al., 2005), to DEHP (Lee et al., 2009; Gentry et al., 2011; Pocar et al., 2012) or to VZ (Bayley et al., 2003; Elzeinova et al., 2008; Eustache et al., 2009; Quignot et al., 2012). In the present study, males exposed to E_2 did not produce any sperm (Hatef et al., 2012b, c). Lahnsteiner et al. (2006) reported that E_2 reduces sperm volume at ≥1 ng/L. Sperm volume is determined by seminal plasma secretion and number of sperm released from testis into the sperm duct. These phenomena are regulated by androgens (particularly 11-KT). Therefore, observed decreases in 11-KT level of fish exposed to BPA, DEHP or VZ (see below) suggest the hypothesis that all of these compounds act on testis causing reduction in sperm production by 11-KT biosynthesis disruption leading to partially or absolutely inhibition of spermatogenesis.

SPERM MOTILITY AND VELOCITY

The present study shows decrease of both sperm motility and velocity in fish exposed to 0.6–11 µg/L or 0.2–20 µg/L BPA (Hatef et al., 2012a, d) which consistent with previous study on fish and mammals (Lahnsteiner et al., 2005; Chitra et al., 2003; Salian et al., 2009). To our knowledge, this is first study that shows decrease of sperm motility and velocity in fish exposed to DEHP and VZ (Hatef et al., 2012b, c). Adverse effects of DEHP and VZ have been previously shown in mammals at environmentally relevant concentration (Eustache et al., 2009; Vo et al., 2009; Gentry et al., 2011). Evaluation of both sperm motility and velocity is a critical endpoint of EDCs because sperm fertility is highly depends on

these parameters in fish (Linhart et al., 2005, 2008). Therefore, these results provide valuable reason for observed decrease in sperm fertility of fish exposed to DEHP (Uren-Webster et al., 2010) and to VZ (Martinovic et al., 2008). In addition to these parameters, cytoskeletal proteins in sperm could be considered as indicators for qualification of sperm (Peknicova et al., 2002; Meeker et al., 2010).

Although, these data shows effects of these compounds on sperm motility, but their modes of action are still unknown. It is seems that EDCs disrupt sperm maturation, which is an important step for acquisition of potential for sperm activation (Morisawa and Morisawa, 1986; Alavi and Cosson, 2006). Therefore, one of potential target is germ cells, because sperm maturation is controlled by progestagens, particularly 17,20β-P biosynthesis in sperm cells induced by LH release from pituitary (Miura et al., 1992; Nagahama, 1994). Another target might be addressed to sperm signaling cascade after release from sperm duct into the aquatic environment. It is known that freshwater fish sperm is immotile in the seminal plasma (Morisawa and Morisawa, 1986). The motility of sperm is triggered by a hypo-osmotic signal, which triggers intracellular sperm signaling (Alavi and Cosson, 2006; Cosson, 2010). Therefore, further studies on the effects of EDCs on sperm morphological and intracellular signaling cascades required for sperm activation will provide valuable information for better understanding of EDCs effects on sperm motility. Previously, both *in vivo* and *in vitro* studies showed damage to sperm morphology, particularly flagellar structure, when fish or sperm were exposed to tributyltin and mercury chloride (McAllister and Kime, 2003; Hatef et al., 2011)

Concerning decrease of sperm velocity, the observed adverse effects of EDCs might be addressed to ATP content and regeneration of ATP; suggesting that mitochondria are targets of VZ (Hatef et al., 2011; Rurangwas et al., 2002). Sperm velocity in fish is highly depending on flagellar beating, which is ATP-dependent activity (Alavi et al., 2009; Cosson, 2010; Butts et al., 2010). We have recently reported damage to sperm mitochondria and reduction in ATP in fish sperm exposed to $HgCl_2$, *in vitro* (Hatef et al., 2011).

Taken together, these data indicate that EDCs influence reproductive performance in fish at environmentally relevant concentrations, which cause male infertility. Among parameters evaluated in the present study, these results recommend that sperm production, motility and velocity could be considered as a good biological indictor in environmental risk assessment. In contrast to estrogenic compound (E_2), GSI may not be the main endpoint for toxicity of BPA, DEHP or VZ.

ENDOCRINE DISRUPTING CHEMICALS AND TESTICULAR STEROIDOGENESIS, *IN VIVO*

TESTICULAR ANDROGEN BIOSYNTHESIS

Endocrine disruption occurs when sex steroid concentrations are either high or low; or hormonal balance change (Quignot et al., 2012). The present study shows novel data in decrease of androgens in fish exposed to environmentally relevant concentrations of BPA (0.6–11 µg/L), VZ (800 µg/L) and DEHP (1–100 µg/L) (Hatef et al., 2012a, b, c). In case of BPA, decrease of T has been previously reported, but the effective dose of BPA was extremely high, for example in male common carp exposed to 1000 µg/L BPA for 14 days (Mandich et al., 2007), in juvenile turbot exposed to 59 µg/L BPA for 21 days (Labadie and Budzinski, 2006) and in larvae brown trout exposed to 50 µg/L BPA for 63 days (Bjerregaard et al., 2008). In case of DEHP, one study shows T decrease in male fathead minnow after exposure to mixture of DEHP and an herbicide linuron (Crago and Klaper, 2012). All of these data suggest to hypothesize that BPA, DEHP and VZ act as anti-androgen at environmentally relevant concentrations similar to mammals (Lee et al., 2006, 2009; Akingbemi et al., 2004; Howdeshell et al., 2007; Culty et al., 2008; Hannas et al.,

2011). The mode of action of each compound may differ, but one possible general reason is due to direct effects of BPA and VZ on HPG axis to control androgenesis. In this context, BPA and VZ exhibits AR antagonistic action, therefore they can decrease androgen biosynthesis in testis via competitive binding to AR and inhibiting positive feedback at the level of hypothalamus-pituitary (Akingbemi et al., 2004; Sharpe et al., 2004; Martinovic-Weigelt et al., 2011). But in case of DEHP, our further analysis showed no differences in transcript mRNA of AR (see below), which suggest independent-AR mode of action which cause disruption in androgenesis via inhibition of LH secretion from pituitary. This hypothesis needs to be examined.

Similar to BPA, DEHP and VZ, 11-KT was also decreased in fish exposed to E_2 for one month (Hatef et al., 2012b, c). This data consistent with other studies and suggest different modes of action of E_2 to decrease androgens via direct effects on hypothalamus and pituitary to inhibit LH secretion (Dæhlin et al., 1986; MacLatchy et al., 1997; Sharpe et al., 2007; Scott et al., 2009; Hannas et al., 2011).

Among EDCs tested in the present study, 11-KT was increased only in goldfish exposed to 100 µg/L VZ (Hatef et al., 2012c), which consistent with previous studies in fathead minnow (Makynen et al., 2000; Martinovic et al., 2008) and rat (Quignot et al., 2012). Considering to decrease of 11-KT observed at 800 µg/L VZ, these data show significant dose-dependent effects of VZ on steroidogenesis (11-KT). At 100 µg/L VZ, disruption in androgenesis occurs because of blockage of T negative feedback in hypothalamus and pituitary (Liebmann and Matsumoto, 1990).

TESTICULAR E_2 BIOSYNTHESIS

E_2 concentration did not change in fish exposed to BPA (0.6–11 µg/L), DEHP (1–100 µg/L), VZ (100–800 µg/L) (Hatef et al., 2012a, b, c), which confirm their non-estrogenic mode of action. In contrast, E_2 increase in fish exposed to E_2 (5 µg/L) which confirms its estrogenic mode of action. Observed increase of E_2 confirms estrogenic activity of these compounds, which is mediated by ER-mediated system (see below) leading to induction of Vtg production in liver (Hatef et al., 2012; Bolger et al., 1998; Ishibashi et al., 2001; Seki et al., 2006; Nelson and Habibi, 2010). Induction of Vtg observed at 11 µg/L BPA observed in the present study also suggests estrogenic activity of BPA at high environmentally relevant concentrations (Hatef et al., 2012a). Different studies show Vtg induction in a dose- and time-dependent manner. For example, increase in Vtg has been reported in adult common carp (Mandich et al., 2007) and medaka (Kang et al., 2002; Ishibashi et al., 2005) at 1000 ug/L BPA after 14 and 21 days exposure, respectively. Lindholst et al. (2000) observed Vtg induction in rainbow trout exposed to 500 µg/L BPA for 12 d. In fathead minnow, significant induction in Vtg production has been observed following 43 or 71 d after exposure to 460 or 160 µg/L BPA (Sohoni et al., 2001). The differences among studies could be addressed to BPA uptake rates, species-specific ER binding affinities, fish species, age, maturity stage and exposure period (Lindholst et al., 2000; Crain et al., 2007).

ENDOCRINE DISRUPTING CHEMICALS AND ALTERATIONS IN SEX STEROID MEDIATING GENES

In the present study, alternations in mRNA transcripts of sex steroid receptors, Vtg and steroidogenesis mediating genes encoding steroidogenic enzymes were studied in fish exposed to BPA, DEHP and E_2 to understand their modes of action at molecular level.

The present study shows StAR mRNA transcript decrease in testis of fish treated with BPA (0.2 µg/L), DEHP (1–100 µg/L) and E_2 (5 µg/L) (Hatef et al., 2012b, d) which consistent with previous study on mammals (Borch et al., 2006; Howdeshell et al., 2007; Culty et al., 2008; Vo et al., 2009; Hannas et al., 2011); data about fish is very rare. Sharpe et al. (2007) reported decrease in StAR and androgen concentration

in goldfish exposed to β-sitosterol after 5 months of exposure. These data suggest that the observed decrease of 11-KT in fish exposed to BPA or DEHP might be related to disruption in transfer of cholesterol to the inner mitochondrial membrane. This is first key step in steroid biosynthesis that provides substrates for P450scc to produce pregnenolone (Stocco and Clark, 1997). Therefore, BPA (0.2 μg/L), DEHP and E_2 exhibit similar action to alter StAR mRNA level linked to inhibition of androgenesis (Hatef et al., 2012b, d). Moreover, StAR mRNA transcript was unchanged in brain of fish exposed to DEHP, but decreased in E_2 treated fish. This is interesting because StAR has been detected in the adrenal and testis and was specifically confined to only the steroid-producing cells of those tissues (Pescador et al., 1996; Clark et al., 1995;).

Similar to E_2, androgen receptor (AR) mRNA transcript was increased in fish exposed to 20 μg/L, but unchanged in DEHP treated fish. This is a strong evidence for role of AR in testicular androgenesis. Both BPA (20 μg/L) and E_2 decrease androgen production via competitive binding to AR, which alter hypothalamus-pituitary regulatory functions in androgenesis.

In contrast to DEHP with no affinity to estrogen receptor (ERα) (Hatef et al., 2012b), BPA showed affinity to bind to ERs in the brain, testis and liver similar to that of E_2 (Hatef et al., 2012b, d). The most significant changes were observed when fish were exposed to BPA at 20 μg/L which concurrent with increase in Vtg mRNA transcript (Hatef et al., 2012d). Similar increase in Vtg mRNA transcript when fish were exposed to BPA has been previously reported (Tabata et al., 2001; Yamaguchi et al., 2005; Huang et al., 2010) leading to increase the Vtg concentration in serum (Hatef et al., 2012a).

In mammals and fish, BPA binding to ERs has been frequently reported but results are somewhat contradictory regarding to subtype of ER. In the present study, mRNA transcripts of all ER subtypes (ERα, ERβ1 and ERβ2) were increased. In tilapia, BPA increased ERα and ERβ1 mRNA transcripts (Huang et al., 2010). In a hermaphroditic fish BPA increased ERα mRNA transcript, while ERβ mRNA transcript unchanged (Seo et al., 2006). In the rat brain, BPA stimulated ERβ mRNA transcript without affecting ERα, while ERβ and ERα mRNA transcripts were decreased and increased in testis, respectively (Akingbemi et al., 2004). Contributing factors that may explain observed variability in ERs and tissue-specific transcription response to BPA include differences in exposure time, concentration, mode of administration as well as differences in affinity of BPA to various ERs (Crain et al., 2007; Wetherill et al., 2007; Nelson and Habibi, 2008, 2010). These data suggest estrogenic mode of action of BPA at high dose through direct ER binding or indirect actions of endogenous E_2 (Crain et al., 2007).

The present study showed that brain-specific P450 aromatase (CYP19b) only increases in fish exposed to E_2 (Hatef et al., 2012b). These results are in agreement with previous studies in which exposure to estrogenic compounds caused up regulation of CYP19 mostly, but failed to modulate testis-specific P450 aromatase (CYP19b) (Kishida et al., 2001; Kazeto et al., 2004; Guyon et al., 2012). This differential responsiveness of P450 aromatase to estrogenic compounds is consistent with the fact that estrogen-responsive elements are identified in the brain CYP19 promoter region of fish species, but not in testis CYP19 (Callard et al., 2001). In contrast, we observed increase in both CYP19a and CYP19b mRNA transcripts in fish exposed to 20 μg/L BPA which are in agreement with previous works on medaka and zebrafish exposed to high concentration of BPA (Kishida et al., 2001; Min et al., 2003; Lee et al., 2006). Increase of mRNA transcript of P450 aromatase either in brain or testis provides evidences for estrogenic mode of action of the examined EDCs because these genes are responsible to convert androgens to estrogens. Therefore, data of the present study confirms further data regarding estrogenic modes of action of BPA at 20 μg/L (Hatef et al., 2012d).

Analysis of transcription of CYP1A mRNA suggests that DEHP and E_2 may act by suppressing metabolizing activity in liver causing toxic effects of DEHP and E_2 to disrupt HPG function in fish reproduction (Hatef et al., 2012b). This is a detoxifying gene transcribed spatially in liver of fish (Olsvik et al., 2007). It is a pollutant-metabolizing enzyme and its suppression attribute to high E_2 level in plasma

(Elskus, 2004). This study shows decrease of CYP1A mRNA transcript in liver of fish exposed to 100 µg/L DEHP and E_2 (Hatef et al., 2012b) which confirm previous results showing inhibitory function of E_2 on CYP1A enzyme activity (Elskus, 2004; Hasselberg et al., 2004).

CONCLUSION AND REMARKS

In conclusion, the present study showed that BPA, DEHP and VZ cause endocrine disruption in fish leading to alternations in fish reproductive performance. Among these compounds, BPA at low dose (0.2–11 µg/L), DEHP at 1–100 µg/L and VZ at 800 µg/L exhibited anti-androgenic modes of action, which inhibit testicular androgenesis. Our results showed that these alternations are mediated by decrease of StAR mRNA transcript which lead to decrease of substrate for steroidogenesis. In this context, further studies are required to investigate alternations in transcripts of genes encoding key elements enzymes in steroidogenesis pathway. Moreover, neuroendocrine functions are largely unknown. Further studies should also consider roles of GnRH, FSH and LH in regulation of testicular spermatogenesis when fish are exposed to these EDCs.

Among EDCs examined in the present study, BPA at 11 µg/L, similar to E_2, showed increase in Vtg production which further analysis on fish exposed to 20 µg/L showed estrogenic modes of action of BPA mediated by ER subtypes and P450 aromatase in the testis. Neither DEHP nor VZ exhibited estrogenic effect.

DEHP mode of action was shown to be sex steroid receptor independent mechanism. This is different from that of BPA which influences reproductive functions through alternations in ER or AR. In case of VZ, we did not study alternations in transcripts of sex steroid mediating genes. According to literature, it was suggested that VZ acts via AR antagonist mode of action which inhibit androgen functions during steroidogenesis. This hypothesis should be studied in future.

Case study on evaluation of CYP1A transcript which encode metabolic enzymes for DEHP in liver suggests that toxicity of EDCs may occur through disruption in metabolism in liver, but little is known about BPA and VZ and should be considered in further studies.

In all cases, sperm quality was decreased in fish exposed to BPA, DEHP or VZ. These alternations were associated with disruption in testicular steroidogenesis mediated by sex steroid mediating genes. In this regard, less is known about alternations in progesterones which regulate sperm maturation as well as intracellular signaling such as cAMP and pH. Therefore, future directions should be given to role of LH to regulate progesterone production in sperm cells as well as potentiality of sperm for activation.

In vitro using BPA and $HgCl_2$, studies suggested disruption in energetics and damage to sperm cells for toxicity of EDCs. In this context, toxicity occurs at concentrations higher than environmental level.

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ENGLISH SUMMARY

Sperm Functions Impairments and Steroidogenesis Transcriptomic Alternations in Fish Exposed to Endocrine Disrupting Chemicals

Azadeh Hatef

The endocrine disrupting chemicals (EDCs) are a large number of natural or synthetic compounds that cause adverse effects on reproductive performance and male fertility by interfering with endocrine system via receptor mediated or receptor independent mechanisms. Most studies on reproductive dysfunctions in aquatic animals have been focused on estrogenic EDCs causing feminization in fish, comparatively little attention has been given to those might interact with androgens, undoubtedly due to the fact that the functions of androgen receptors (AR) are largely unknown in reproduction. Moreover, the literature on EDCs modes of action is somewhat contradictory. For instance, it has been thought that Bisphenol A (BPA) and Di-(2-ethylhexyl)-phthalate (DEHP) exhibit estrogenic activities due to their affinities to estrogen receptors (ER), while recent studies show their anti-androgenic mode of action. Although, there are several studies that show effects of these compounds on alternations of reproductive hormones in fish, but there is a lack of information about their mechanisms of toxicity at gene level as well as their potential effects to alter sperm quality as a critical endpoint for male fertility. In the present study, *in vitro* experiments were conducted to understand whether EDCs alter sperm function at environmentally relevant concentration. Then, the fish were exposed to EDCs, *in vivo*, for understanding of the potential toxicity of EDCs as well as their modes of action that provide biological indictors for environmental risk assessments.

Results obtained from *in vitro* experiments showed that sperm motility and velocity were reduced after activation in presence of 34×10^3 µg/L (125 mM) $HgCl_2$ and 228×10^3 µg/L (1 mM) BPA. The Vinclozolin showed no effect on sperm motility at concentrations up to 20×10^3 µg/L. These results suggest that toxic potency of EDCs on sperm function is occurred at higher concentrations than reported in the aquatic environments. However, due to bioaccumulation of EDCs in reproductive organs, the $HgCl_2$ toxicity was studied in incubated sperm in $HgCl_2$. Results showed decrease in sperm motility and velocity at 85 and 850 µg/L $HgCl_2$, respectively, following 3 h incubation of sperm in immobilizing medium containing the same concentrations of $HgCl_2$, indicating stronger effects of $HgCl_2$. In this context, $HgCl_2$ acts on sperm through decreasing ATP content and disruption of the plasma membrane, mitochondria, axoneme function causing disruption of sperm motility and energetics.

In contrast, *in vivo* experiments showed that sperm motility and velocity were decreased in fish exposed to 0.2–20 µg/L BPA for 1–3 months, and to 10–100 µg/L DEHP or 400–800 µg/L VZ for 1 month. These results suggest that EDCs causing male infertility through disruption in spermatozoa maturation, which is an important step for acquisition of potential for sperm activation. Decrease in sperm velocity might be an indictor for disruption in sperm ATP content as well as morphology of sperm flagella. Moreover, in these experiments, sperm volume showed significant decrease in fish exposed to 0.2–20 µg/L BPA for 3 months and to 10–100 µg/L DEHP or 800 µg/L VZ for 1 month. These data suggest that EDCs examined in the present study disrupt spermatogenesis or sperm release from testis, which is regulated by androgens under physiological condition.

Results showed decrease of androgens (both Testosteron and 11-KetoTetosteron) in fish exposed to 0.6–11 µg/L BPA. In fish exposed to DEHP, 11-KT was reduced at all examined concentrations (1, 10 and 100 µg/L). In case of VZ, increase and decrease in 11-KT was observed at 100 and 800 µg/L, respectively. Therefore, these results suggest that obseved reduction in sperm quality might be related to disruption in testicular androgenesis. No efects of BPA, DEHP or VZ were observed on E_2 level in the blood plasma

which indiacte non-esrtrogenic activity of these EDCs, except for BPA which induced vitellogenin (Vtg) level in fish exposed to 11 μg/L for 1 month.

To understand mechanism of steroidogenesis disruption, alternations in mRNA transcript of ER subtypes, AR, and Vtg were analysed using quantitative real time PCR in two experiments where fish were exposed to BPA and DEHP. In this context, mRNA transcript alterations were studied in the following genes encoding steroidogenic enzymes; steroidogenic acute regulatory protein (StAR) that control transfer of cholesterol to the inner mitochondrial membrane, cytochrome P450 aromatase (CYP19b in brain and CYP19a in testis) that convert androgens to estrogens, and cytochrome P450 1A1 (CYP1A) in liver that is responcible for the biotransformation of xenobiotic compond.

Transcript of StAR mRNA was reduced in fish exposed to 0.2 μg/L BPA and to 1–100 μg/L DEHP. Decrease in CYP1A mRNA transcript was observed in fish exposed to 100 μg/L DEHP. These data indicate that decrease in substrate for steroidogenesis and increase in toxicity of EDCs are key elements due to disruption in transfer of cholesterol to the inner mitochondrial membrane in steroidogenic cells in testis and metabolism of EDCs in liver, respectively.

Bisphenol A showed trends in decrease of AR mRNA transcript in testis when fish was exposed to 0.2 μg/L. In contrast, mRNA transcript of ER subtypes in brain, liver or testis, AR in testis, CYP19b in brain, CYP19a in testis, and Vtg in liver were increased in fish exposed to 20 μg/L. In case of DEHP, sex steroid receptors Vtg mRNA transcript were unchanged in fish.

In conclusion, results shown in the present stduy indicate adverse effects of EDCs on reproductive performance in male fish associated with disruption in testicular steroidogenesis mediated by alternations in mANA transcription of sex steroid receptors, Vtg and sex steroid mediting genes. The EDCs effects on sperm function might be related to dirusption in sperm maturation as well as intracellular signaling and ATP contents required for sperm activation.

CZECH SUMMARY

Vliv endokrinních disruptorů na funkčnost spermií a změny ve steroidogenezi transkriptomik u ryb

Azadeh Hatef

Endokrinní disruptory (EDCs) představují velké množství jak přírodních tak syntetických látek, které mají negativní dopad na reprodukční výkonnost a plodnost u samců tím, že manipulují s endokrinním systémem prostřednictvím receptorů nebo ovlivňují mechanismy zprostředkované receptory. Většina studií na reprodukční dysfunkce u vodních organismů byla zaměřena na estrogenní endokrinní disruptory, které způsobují feminizaci u ryb, a jen malá pozornost byla věnována androgenním EDCs nepochybně díky tomu, že funkce androgenních receptorů (AR) s dopadem na reprodukci jsou do značné míry neznámé. Navíc dostupná literatura o EDCs je poněkud nejednotná a rozporuplná. Příkladem by mohly být látky bisfenol A (BPA) a di-(2-ethylhexyl)-ftalát (DEHP), které vykazují estrogenní aktivitu díky jejich afinitě k estrogenním receptorům (ER), zatímco nedávné studie ukazují jejich antiandrogenní účinek. Přestože existuje několik studií, které dokazují, že tyto látky mají vliv na střídání pohlavních hormonů u ryb, není však dostatek informací o jejich toxickém účinku na genové úrovni s možností dopadu na změnu kvality spermií jakožto kritického koncového bodu pro mužskou plodnost. V této studii byly *in vitro* experimenty navrženy a provedeny pro pochopení působení EDCs, o koncentracích shodných s koncentracemi v životním prostředí, na změny funkcí spermií. Poté byly ryby vystaveny působení EDCs *in vivo*, pro pochopení potenciální toxicity EDCs, které poskytují biologické indikátory určené pro hodnocení rizik v životním prostředí.

Výsledky získané z *in vitro* experimentů ukázaly, že pohyblivost a rychlost pohybu spermií byla po aktivaci snížena za přítomnosti 34×10^3 μg/L (125 mM) $HgCl_2$ a 228×10^3 μg/L (1 mM) BPA. Vinklozolin (VZ) neměl žádný účinek na pohyblivost spermií v koncentracích až 20×10^3 μg/L. Tyto výsledky naznačují, že toxické účinky EDCs na funkčnost spermií se vyskytly pouze u vyšších koncentrací, které se v běžných přírodních podmínkách nevyskytují. Nicméně, z důvodu bioakumulaci EDCs v reprodukčních orgánech se spermií záměrně inkubovaly v časové řadě různě koncentrované $HgCl_2$. Výsledky ukázaly na pokles pohyblivosti a rychlosti pohybu spermií po inkubaci v 85 a 850 μg/L $HgCl_2$. Toxicita se projevila zejména po 3 h inkubace spermií v imobilizačním médiu, které obsahovalo stejné koncentrace $HgCl_2$, což naznačovalo, že silnější účinky $HgCl_2$ závisí na inkubační době. V této souvislosti $HgCl_2$ působí na spermie prostřednictvím snížení obsahu ATP, narušuje plazmatickou membránu, mitochondrie a funkci bičíku, což způsobuje změny v pohyblivosti spermií a jejich energetice.

Naproti tomu, *in vivo* experimenty na mlíčácích ukázaly, že pohyblivost spermií a jejich rychlost pohybu se snížila u ryb, které byly vystaveny působení EDCs o koncentracích: 0,2–20 μg/L BPA po dobu 1–3 měsíců, a 10–100 μg/L DEHP, nebo 400–800 μg/L VZ po dobu 1 měsíce. Tyto výsledky naznačují, že EDCs způsobují neplodnost u samců z důvodu narušení dozrávání spermií, což je nezbytný krok pro získání budoucích aktivních spermií. Snížení rychlosti pohybu spermií může být ukazatelem pro narušení obsahu ATP u spermií, stejně jako morfologie bičíku u spermií. Navíc, tyto experimenty poukázaly na výrazné snížení počtu spermií u ryb, které byly vystaveny působení BPA o koncentraci 0,2–20 μg/L po dobu 3 měsíců a DEHP o koncentraci 10–100 μg/L nebo VZ o koncentraci 800 μg/L po dobu 1 měsíce. Tyto data naznačují, že EDCs použité v této studii narušují spermatogenezi a spermiaci a tím i fyziologickou hladinu androgenů. Výsledky ukázaly na pokles androgenů (jak testosteronu – T tak i 11-ketotestosteronu – 11-KT) u ryb, které byly vystaveny působení BPA o konc. 0,6–11 μg/L. U ryb, které byly vystaveny účinku DEHP, došlo ke snížení 11-KT ve všech zkoumaných koncentracích (1, 10 a 100 μg/L). V případě VZ, byl nárůst a pokles 11-KT pozorován hlavně při vystavení účinku VZ

o konc. 100 a 800 µg/L. Výsledky naznačují, že pokles kvality spermií může být zapříčiněn narušením androgeneze v testes. Žádný účinek nebyl pozorován po působení BPA, DEHP nebo VZ v krevní plazmě na úrovni E_2 a naznačuje tak neestrogenní účinek těchto EDCs, s výjimkou BPA, který u ryb, jež byly vystaveny jeho působení o konc. 11 µg/L po dobu 1 měsíce, způsobil indukci vitellogeninu (Vtg).

Pro pochopení narušení mechanismů steroidogeneze, změn v přepisu mRNA u ER, AR a Vtg byly provedeny dva pokusy, ve kterých byly ryby vystaveny působení BPA a DEHP a experimenty byly anylyzovány pomocí kvantitativní real-time PCR (qPCR). V této souvislosti byly studovány změny v přepisu mRNA u genů kódujících enzymy steroidů. V prvé řadě šlo o steroidní akutní regulační protein (StAR), který řídí přenos cholesterolu z vnější do vnitřní mitochondriální membrány. Dále byly studovány cytochrom P450 aromatázy (v mozku CYP19b a v testes CYP19a), které převádí androgeny na estrogeny a cytochrom 1A1 P45 (CYP1A) v játrech, který je zodpovědný za biotransformaci xenobiotik. Transkripce StAR mRNA byla nižší u ryb, které byly vystaveny působení BPA o konc. 0,2 µg/L a DEHP o konc. 1–100 µg/L. Pokles v přepisu mRNA byl také pozorován u CYP1A, kde byly ryby vystaveny DEHP o konc. 100 µg/L. Tyto údaje ukazují, že pokles v substrátech pro steroidogenezi a naopak zvýšení toxicity EDCs jsou klíčovými prvky, v důsledku kterých dochází k přerušení přenosu cholesterolu z vnější do vnitřní mitochondriální membrány, a to hlavně u steroidních buněk v testes a metabolismu EDCs v játrech.

Bisfenol A o konc. 0,2 ug/L u ryb opakovaně prokázal pokles v přepisu mRNA AR v testes. Na rozdíl od mRNA transkriptu ER subtypů v mozku, játrech nebo testes, AR v testes, CYP19b v mozku, CYP19a v testes a Vtg v játrech u ryb došlo k jejich zvýšení po působení BPA o konc. 20 ug/L. V případě působení DEHP nebyly u ryb pozorovány žádné změny v přepisu mRNA Vtg pohlavních steroidních hormonů.

Závěrem lze říci, že výsledky z uvedené studie prokazují, že EDCs působí negativně na reprodukční schopnosti u mlíčáků a jsou spojené s narušením testikulární steroidogeneze zprostředkované pomocí změn v přepisu mRNA u pohlavních receptorů, Vtg a genů pohlavních hormonů. Negativní účinek EDCs na funkčnost spermií může být spojen s jejich narušením v době jejich zrání, stejně jako s intracelulárním přenosem signálu a obsahem ATP potřebného pro jejich aktivaci.

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A journey is easier when you travel together. Interdependence is certainly more valuable than independence.

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LIST OF PUBLICATIONS

PEER-REVIEWED JOURNALS WITH IF

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BOOK CHAPTER

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TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Azadeh Hatef
Research department	2008–2012 Laboratory of Reproduction physiology FFPW
Daily supervisor	Prof. Dipl.-Ing. Otomar Linhart, D.Sc.
Supervisor	Prof. Dipl.-Ing. Otomar Linhart, D.Sc.
Period	From October 1, 2008 to September 12, 2012

Ph.D. courses	**Year**
Fish reproduction	2009
Pond aquaculture	2010
English language	2010
Applied hydrobiology	2011
Ichthyology and systematics of fish	2011
Basics of scientific communication	2011
Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2008 2010 2011 2012
International conferences	**Year**
The 2nd International Workshop on the Biology of Fish Gametes, Valencia, Spain, September 9–11. (Poster presentation)	2009
The 2nd National Conference of Fisheries Sciences and Aquatic Animals, Lahijan, Iran, May 10–12. (Oral presentation)	2011
Diversification in Inland Finfish Aquaculture, Pisek, Czech Republic, May 16–18. (Oral & Poster presentation)	2011
The 3rd International Workshop on the Biology of Fish Gametes, Budapest, Hungary, September 7–9. (Oral & Poster presentation)	2011
The 9th Symposium on Reproductive Physiology of Fish, Cochin, India, August 9–14. (Poster presentation)	2011
AQUA 2012 – Global Aquaculture – Securing Our Future. Prague, Czech Republic, September 1–5. (Oral presentation)	2012
Foreign stays during Ph.D. study at FFPW and RIFCH	**Year**
May–July 2010: Prof. Hamid R. Habibi, Ph.D. Dept. of Biological Sciences, University of Calgary, Alberta, Canada. Topic: Modulation of androgen and estrogen mediting genes and testiculr response in goldfish exposed to bisphenol A. Period of stay: 2 months	2010

October–November 2010 & September 2011: Prof. Oliana Carnevali, Ph.D. Dept. of Marine Biology, Marche Polytechnic University, Ancona, Italy. Topic: Modes of action of phthalate on male reproductive physiology in goldfish. Period of stay: 2 and ½ months	2010–2011
October 2011-February 2012: Associ. Prof. Maria S. Sepulveda, Ph.D. Dept. of Forestry and Natural Resources, Purdue University, West Lafayette, IND, USA. Topic: *In vivo* assays for modeling effects of endocrine disrupting chemicals on fish gonadal development and sex differentiation in Medaka. Period of stay: 4 months	2011–2012

CURRICULUM VITAE

PERSONAL INFORMATION

Name:	**Azadeh Hatef**
Title:	**Master of Science (M.Sc.)**
Born:	May 14, 1979, Langroud, Iran
Nationality:	Iranian

PRESENT POSITION

Ph.D. student at the University of South Bohemia in České Budějovice (USB), Faculty of Fisheries and Protection of Waters (FFPW, www.frov.jcu.cz), Research Institute of Fish Culture and Hydrobiology (RIFCH), Laboratory of Reproduction Physiology, Vodňany, Czech Republic.

EDUCATION

1998–2002	B.Sc., Natural Resources Sciences Engineering – Fisheries Sciences, University of Agricultural Sciences and Natural Resources, Gorgan, Iran.
2003–2006	M.Sc., Fishery (Fish Culture and Breeding), Tehran University, Tehran, Iran.
2008 – present	Ph.D., USB, FFPW, RIFCH, České Budějovice, Czech Republic.

RESPONSIBLE LEADER OF PROJECTS

Grant Agency of the University of South Bohemia GAJU 033/2010/Z (2010). Mechanisms of action of Bisphenol A on steroidogenesis using gene expression of vitellogenin, StAR, P450scc, 3beta-HSD and CYP19 in male fish. (leader Azadeh Hatef, M.Sc.)

AWARDS

2011	Best Poster presentation chosen by Scientific Committee "Diversification in Inland Finfish Aquaculture, Pisek, Czech Republic, May 16–18, 2011".
2012	"Highlighted Article" – chosen by Editor-in-Chief of Ecotoxicology and Environmental Safety for outstanding scientific quality and relevance.

FOREIGN STAYS DURING PH.D. STUDY AT FFPW AND RIFCH

May – July 2010	Prof. Hamid R. Habibi, Ph.D., Dept. of Biological Sciences, University of Calgary, Alberta, Canada.
October – November 2010 and September 2011	Prof. Oliana Carnevalli, Ph.D., Dept. of Marine Biology, Marche Polytechnic University, Ancona, Italy.

October 2011 – February 2012 Associ. Prof. Maria S. Sepulveda, Ph.D., Dept. Forestry and Natural Resouces, Purdue University, West Lafayette, IND, USA.

PH.D. COURSES

Fish reproduction, Applied hydrobiology, Ichthyology and systematics of fish, Pond aquaculture, English language and Basics of scientific communication.

FACULTY OF **FISHERIES** AND **PROTECTION** OF **WATERS**

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