Fakulta rybářství
a ochrany vod
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Jihočeská univerzita
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Environmental contaminants and endocrine associated male infertility in fish

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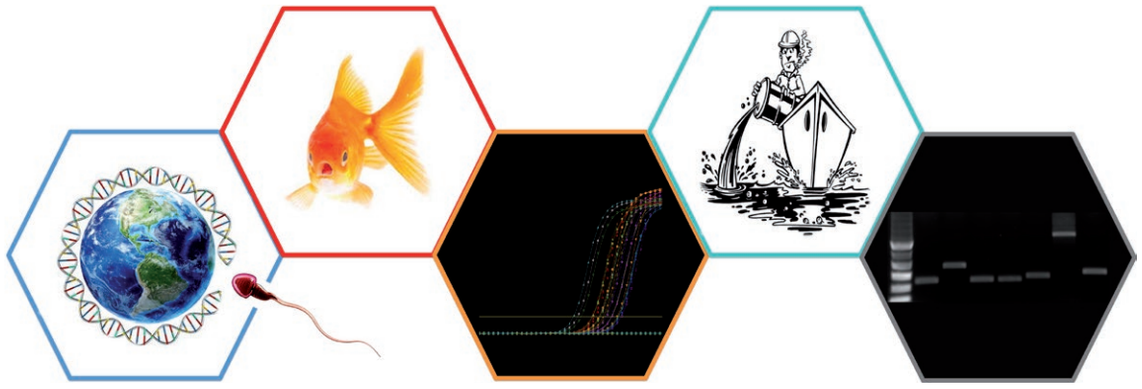

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CONTENT

List of symbols and abbreviations

>	greater than
<	less than
11-KT	11-ketotestosterone
3β-hsd	3-β-hydroxysteroid dehydrogenase
17β-hsd	17-β-hydroxysteroid dehydrogenase
20β-hsd	20-β-hydroxysteroid dehydrogenase
acox1	acyl-CoA oxidase 1
AMH	anti-Mullerian hormone
AR	androgen receptor
AR α	androgen receptor α subunit
ANOVA	one-way analysis of variance
ATP	adenosine triphosphate
°C	centigrade
CA	calcium
cDNA	complementary deoxyribonucleic acid
cAMP	cyclic adenosine monophosphate
cGnRH	chicken gonadotropin-releasing hormone, GnRH type 2
cm	centimeter
CYP11B1	cytochrome p45011β-hydroxylase
CYP17	cytochrome P450 17alpha-hydroxylase/17,20-lyase
CYP19	aromatase or cytochrome p450
CYP19a1b	brain p450 aromatase
C_t	threshold cycle
d	day
dazl	deleted in azoospermia-like
DEHP	di-(2-ethylhexyl)-phthalate
DHP	17α, 20β-dihydroxy-4-pregnen-3-one
dNTP	deoxyribonucleotide
E_2	estradiol
EDCs	endocrine disrupting chemicals
ECs	environmental contaminants
ehhadh	enoyl-CoA, hydratase/3-hydroxyacyl CoA dehyrogenase
ER	estrogen receptor
ER-α	estrogen receptor α subunit
ER-β	estrogen receptor β subunit
ER-γ	estrogen receptor γ subunit
eSRS22	eel spermatogenesis related substance 22
FLU	flutamide
FSH	follicle-stimulating hormone
FSH-β	follicle-stimulating hormone β subunit
FSHR	follicle-stimulating hormone receptor
g	gram
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotropin-releasing hormone

GSI	gonadosomatic index
GTH	gonadotropin
h	hour
hrb	HIV-1 Rev binding protein
HDL	high density lipoproteins
HPT	hypothalamic-pituitary-testis
HSI	hepatosomatic index
IGF-1	insulin-like growth factor 1
l	liter
LDL	low density lipoproteins
LH	luteinizing hormone
LH-β	luteinizing hormone β subunit
LHR	luteinizing hormone receptor
M1	2-{[(3,5-dichlorophenyl)-carbamoyl]oxy}-2-methyl-3-butenoic acid
M2	3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide
mg	milligram
mM	millimoles/liter
min	minute
M-MLV	Moloney Murine Leukemia virus reverse transcriptase
mRNA	messenger ribonucleic acid
nm	nonometer
oligo dT	short sequence of deoxythymine
PCR	polymerase chain reaction
ppar	peroxisome proliferator-activated receptor
qRT-PCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
RNAse	ribonuclease
s	second
sGnRH	salmon gonadotropin-releasing hormone, GnRH type 3
spnr	spermatid perinuclear RNA-binding protein
SEM	standard error of the mean
StAR	steroidogenic acute regulatory protein
T	testosterone
U	units
Vtg	vitellogenin
VZ	vinclozolin
α	alpha
β	beta
γ	gamma
µg	microgram
µl	microliter

CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

Global rates of environment-related reproductive disorders have been increasing over the past 40–50 years. The incidence of low semen quality and testicular germ cell cancer show significant increases in industrialized countries, reflected in an increase in the number of infertile couples (Phillips and Tanphaichitr, 2008; Lund et al., 2009; Toppari et al., 2010). Public concern regarding environment-related reproductive disorders was initially kindled by observations of reduced fertility, birth defects, and sexual development disorders in wildlife (EFSA Scientific Committee, 2013). For over 10 years, the European Union, the European Food Safety Authority, the World Health Organization, and other organizations comprising working groups of experts in endocrinology, risk assessment, and toxicology have conducted research to study environmental contaminants associated fertility in humans and wildlife (http://ec.europa.eu/health/healthy_environments/policy/index_en.htm). These studies have revealed approximately 800 natural and man-made chemicals known or suspected to interfere with the reproductive system (Bergman et al., 2013). However, only a fraction of these chemicals have been investigated with tests capable of identifying fertility effects in an intact organism. To reduce the risk of environment-related infertility in males, it is critical to evaluate the association of exposure to environmental contaminants with reproductive hormones, as key regulators for fertility. To this end, an interdisciplinary approach is needed to identify environmental contaminants that reduce male fertility.

The aquatic ecosystem is at greatest risk from pollutants, since most chemicals will eventually reach rivers, lakes, and oceans as the final repository (Kime, 1998). Similar to humans, frequent evidences shows trends in male reproductive health in fish from wildlife. Since biology of the reproductive system is highly conserved in vertebrates, studies of fish as model organisms provide substantial information to establish a detailed risk assessment and to establish novel or more sensitive fertility endpoints for environment-related reproductive disorders. Evidence of adverse trends in male fertility in laboratory study appears to mirror male reproductive disorders in wildlife. It has been documented that environmental contaminants including steroid hormones, heavy metals, plasticizers, pesticides, herbicides, fungicides and pharmaceuticals alter reproductive endocrine system and cause infertility (Gregory et al., 2008; Ankley et al., 2009; Scholz and Klüver, 2009; Dang et al., 2011; Leet et al., 2011; Mennigen et al., 2011; Page et al., 2011; Waye and Trudeau, 2011; Scholz et al., 2013; León-Olea et al., 2014). However mechanisms through which environmental contaminants disrupt reproductive system might differ among contaminants, be species specific and poorly understood.

2. An introduction to male reproductive system in fish

It is essential to review the reproductive biology of fish before delving into environment associated reproductive disorders. This will provide the basic information to better understand the multiplicity of sites through which environmental contaminants may disrupt male fertility.

2.1. Neuroendocrine and endocrine regulation of spermatogenesis and steroidogenesis

Neuroendocrine and endocrine hormones regulate spermatogenesis in fish. It is known that negative and positive feedback of sex steroids control also neuroendocrine functions of hypothalamus and pituitary (see reviews by Nagahama, 1994; Young et al., 2005; Vizziano et al., 2008; Watanabe and Onitake, 2008; Kah, 2009; Van der Kraak, 2009; Yaron and Levavi-Sivan, 2006).

The Kiss/Gpr54 system integrate environmental cues (e.g. temperature and light) and internal signal to pass information to hypothalamus to release gonadotropin-releasing hormone (GnRH) which stimulates production and secretion of gonadotropins from pituitary via a receptor-mediated mechanism (Khakoo et al., 1994; Melamed et al., 1996; Illing et al., 1999; Madigou et al., 2000, 2002; Zohar et al., 2010). The gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Suzuki et al., 1988a, 1988b, Itoh et al., 1988), stimulate sex steroid production in pathway of steroidogenesis via receptor-mediated pathways. Steroidogenesis is similar across fish species, involves a series of steroidogenic enzymes and takes place in the Leydig cells (Young et al., 2005) (Figure 1). Briefly, a steroidogenic acute regulatory protein (StAR) delivers cholesterol to the inner mitochondrial membrane. Cytochrome P450 side-chain cleavage (P450scc) located at the inner mitochondrial membrane synthesizes pregnenolone from cholesterol. The *P450scc* function is rate-limited by cholesterol availability (Stocco, 2001). Then, cytochrome P450 17-hydroxylase/$C_{17\text{-}20}$lyase catalyses the hydroxylation of pregnenolone to produce 17α-hydroxypregnenolone. Subsequent removal of the two-carbon $C_{20\text{-}21}$ acetic acid residues from 17α-hydroxypregnenolone by *$C_{17\text{-}20}$lyase* leads to production of dehydroepiandrosterone or androstenedione. Pregnene-androstene- and estrene-derivatives can all be further modified into other steroids by 3β, 17β, and 20*β*-hydroxysteroid dehydrogenase (*3β-hsd, 17β-hsd*, and *20β-hsd*) (Miller, 1988, 2005). For synthesis of 11-Ketotestosterone (11-KT), cytochrome P450 11β-hydroxylase and *11β-hsd* catalyze testosterone to 11-KT which is known as a main androgen in fish (Nagahama, 1994). Synthesis of 17*β*-estradiol (E_2) is mediated by cytochrome P450 aromatase which has two isoforms; isoform *a* and *b* expressed in the testis and brain, respectively (Callard et al., 2001; Kishida et al., 2001). Both 11-KT and E_2 are critical for spermatogenesis.

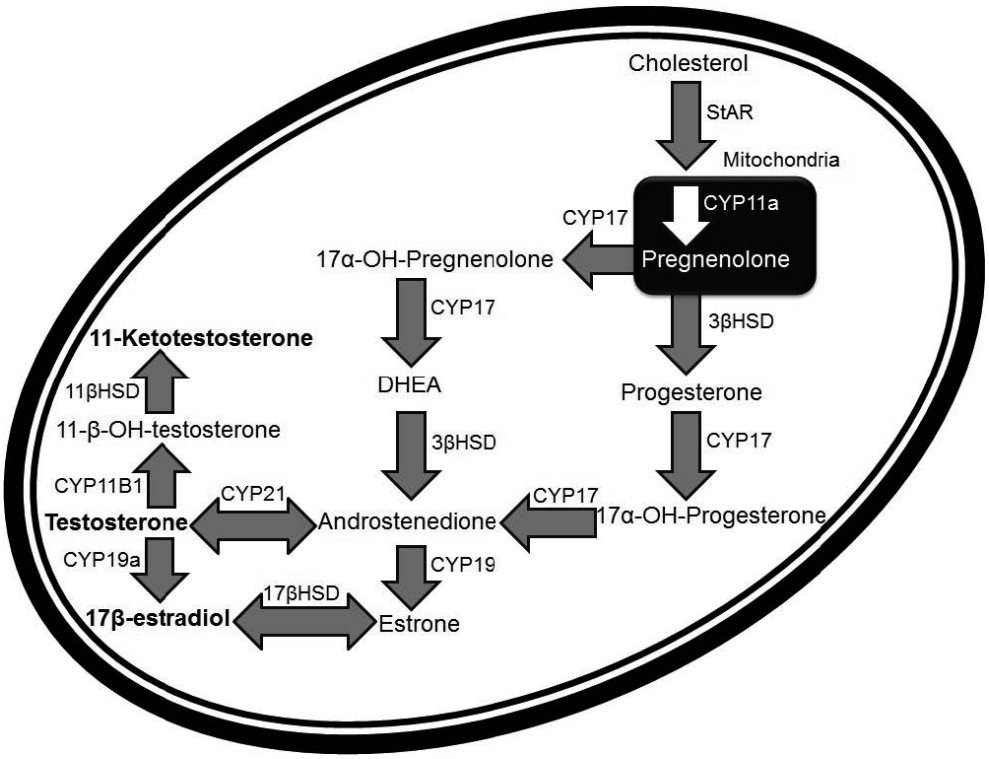

Figure 1. *Generalized overview of testicular steroidogenesis in Leydig cells in fish.*

In spermatogenesis, the FSH stimulates spermatogonial proliferation (mitosis) (Loir, 1999). This process is mediated by release of E_2 from Leydig cells to stimulate the spermatogonial renewal factor (eSRS34) from the Sertoli cells through a receptor (Miura and Miura, 2001). To induce spermatogonial proliferation, FSH stimulates 11-KT secretion from Leydig cells, which stimulates Sertoli cells to produce Activin B and Insulin-like growth factor 1 (Igf-1) (Miura et al., 1995, 1997; Nader et al., 1999). 11-KT is required for spermatogenesis induction, whereas Igf-1 is necessary for its continuation (Miura and Miura, 2003). Activin B induces spermatogonial mitosis, but is unable to induce meiosis because of anti-Mullerian hormone (Amh), which prevents spermatogonial proliferation (Miura et al., 2002). Spermatogonial meiotic division to produce spermatids is induced by 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) secreted from germ cells and spermatids under regulation of 11-KT (Figure 2) (Miura et al., 2006). Spermatozoa released to testicular lobules are immotile with no potential fertilizing ability (Miura et al., 1992). At spawning, spermatozoa maturation is regulated by LH which induces production of progestins (DHP in most fish species) in spermatozoa by activation of 'eel spermatogenesis related substances 22' (eSRS22) which is homologous to carbonic anhydrase type II (CA II) leading to increase in the seminal plasma pH and elevation in cAMP content of spermatozoa (Ueda et al., 1984; Thomas and Trant, 1989; Miura et al., 1992).

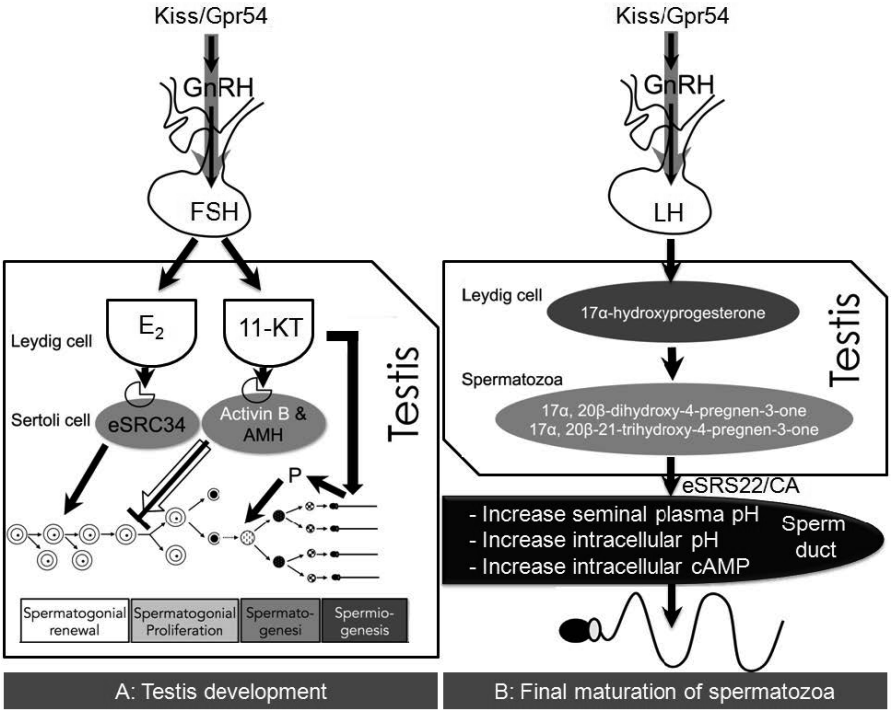

Figure 2. *Neuroendocrine and endocrine regulation of spermatogenesis and sperm maturation in fish during testis development (A) and final maturation of spermatozoa (B).*

2.2. Male fertility indicators in fish

Successful fertilization depends on synchronization of release of spermatozoa from males and ova from females (Kudo, 1991; Coward et al., 2002; Kinsey et al., 2007). Spermatozoa must penetrate the egg through the micropyle to fertilize it during the short motile phase. Besides, it has been shown that fertilization success positively correlates with sperm production per ejaculate which is usually measured by counting, and expressed as number of spermatozoa per ml or total number of spermatozoa (Suquet et al., 1992; Rurangwa et al., 1998; Tvedt et al., 2001; Rideout et al., 2004; Kaspar et al., 2007). Fish spermatozoa are immotile in the seminal plasma and the sperm duct. Motility is triggered upon discharge into the aquatic environment. In most freshwater fish, the key factor preventing spermatozoon motility in the reproductive organ is osmolality and a hypo-osmotic signal is necessary for initiation of sperm motility (Alavi and Cosson, 2006; Morisawa, 2008). Fish spermatozoon motility and velocity decrease rapidly after initiation of motility (Alavi and Cosson, 2005; Cosson, 2010). Short duration and rapid decrease of spermatozoon motility and velocity are associated with considerable, but not complete, depletion of ATP (Ingermann, 2008). Spermatozoon motility and velocity are crucial determinants of male fertility in fish. Several studies have shown that there are positive correlations between spermatozoa motility/velocity and fertilization rate (Alavi et al., 2008).

Overall, all reproductive hormones directly or indirectly control testis development and sperm maturation in fish. Any interfere with neuroendocrines or/and endocrines regulator of reproduction may lead to male infertility. Environmental contaminants which are distributed widely in aquatic environment have potential to disrupt reproductive pathway and lead to male infertility.

3. Model organism

Goldfish (*Carassius auratus*) are known as a suitable model organism for the neuroendocrine signaling and regulation of reproduction in vertebrates. They are easy to sustain in the laboratory and available commercially. Furthermore, hormonal profiles and neuroendocrine regulatory mechanisms of goldfish reproductive cycle is well characterized. (Popesku et al., 2008). It is easy to collect the blood and the sub parts of brain in goldfish are big enough to recognize in dissection with naked eye. Besides, reproductive endocrine system has been extensively studied in goldfish and almost biochemical and molecular tools for the investigation of neuroendocrine and endocrine are available (Chang et al., 2009, 2012; Habibi and Huggard, 1998; Trudeau, 1997). Additionally, goldfish is a multiple spawner and its reproductive physiology is very sensitive to water quality as previous studies showed that sperm quality factors, reproductive hormones and general/specific metabolites disrupted following 10–20 days of exposure to very low doses of environmental contaminants (Jordan et al., 2011; Hatef et al., 2012).

4. Environmental contaminants

For several decades, classifications of environmental contaminats (ECs) have been done by chemical structure and their sources. Kime (1998) classified ECs into three large groups including heavy metals, pesticides and industrial pollutants. Denslow and Sepulveda (2008) classified persistent organic pollutants, pharmaceutics and sewage effluents and paper mill effluents into self-standing groups. Main problem in classification based on ECs structure and source has arisen with rapid increase of numbers of identified ECs. At present, over 80,000

chemicals are known or suspected to be capable of interfering with hormone synthesis, conversion and action (Bergman et al., 2012). Next generation of classifications has considered mechanisms by which ECs act on endocrine reproductive system. Estrogenic, androgenic and anti-androgenic effects of ECs have been largely demonstrated by identification ECs actions to mimic the effects of endogenous sex steroid hormones or to alter hormone homeostasis (Gregory et al., 2008). Recent advanced state-of-the-art models using molecular and genetic approaches classify ECs based on receptor-mediated or receptor-independent pathways. The receptor-mediated pathway has further classified into ECs with a genomic mode of action or non-genomic mode of actions (Diamanti-Kandarakis et al., 2009). Genomic modes of action of ECs are mediated by ECs binding to intracellular nuclear steroid receptors resulting in changes in their transcriptional activity. In non-genomic pathway, ECs interfere with the effects of sex steroids that act through membrane receptor, resulting in the activation of ion channels or intracellular signal transduction.

5. Selected environmental contaminants in the present study

5.1. Vinclozolin

Vinclozolin (VZ) ([3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine 2,4-dione] trade names: Ronilan, Curalan, Vorlan, Touche) (Figure 3) is a common dicarboximide fungicide widely used for control of diseases caused mainly by *Botrytis cineera* and *Sclerotinia* species in fruits and vegetables (Pothuluri et al., 2000), as well as commonly used in the wine industry (Kelce et al., 1994). Vinclozolin itself is not persistent, its two metabolites, M1 (3-[[[3,5-dichlorophenyl]-carbamoyl]oxy]-2-methyl-3-butenoic acid) and M2 (3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide), display half-lives of more than 180 days and are likely to be highly mobile in the water phase.

People may be exposed to residues of VZ via drinking water or food. In surface waters, VZ has been detected at concentrations of 0.1–2.4 μg/L (Readman et al., 1997; El-Shahat et al., 2003). It has been listed under the European Union priority list of ECs (European Commission, 2001) and in the United States Environmental Protection Agency's final Contaminant Candidate List 3 for products that should be assessed for contamination levels in drinking water (U.S. EPA, 2009). So the amount of VZ has been used significant decrease over the past decades.

In mammals, it has been reported that VZ causes disruption in sexual differentiation, testicular development and sex steroid biosynthesis leading to abnormal sperm (Gray et al., 1994, 1999; Kubota et al., 2003; Veeramachaneni et al., 2006; Loutchanwoot et al., 2008; Elzeinova et al., 2008; Eustache et al., 2009; Auger et al., 2010). Surprisingly, reproductive impairments caused by VZ could be transgenerationally transmitted by an epigenetic mechanism (Anway et al., 2005).

The adverse effects of VZ on reproductive endocrine performance in fish are largely unknown and its mode of action has not been understood and is somewhat contradictory (Table 1). Almost studies have shown no effects of VZ on GSI as well as no incidence of intersex (Makynen et al., 2000; Kinnberg and Toft, 2003; Kiparissis et al., 2003). Makynen et al. (2000) were the first who examined VZ potency to interfere with androgen production in fish. They observed no change in T and 11-KT levels following exposure of fathead minnow to 200 and 700 μg/L for 21 d, respectively, which consistent with further experiments of the same research group (Martinovic et al., 2008). Despite of the lack of significant change in androgen level, same authors reported an increase in mRNA testicular *ar* and luteinizing hormone receptor (*lhr*) and *11 β-hsd*, while mRNA of the gene encoding *lh* remained unchanged (Villeneuve et al., 2007; Martinovic et al., 2008). In fathead minnow, Villeneuve et al. (2007) reported a

dose-dependent increase in the pituitary *fshβ* mRNA, which was significantly different from the control in fathead minnow, exposed to 700 μg/L VZ, however *lh* mRNA levels showed a trend toward decrease at 100-700 μg/L VZ. These observations consistent with VZ effects on androgen production in fathead minnow in which 11-KT is not significantly increased following an *in vivo* exposure (Villeneuve et al., 2007; Martinovic et al., 2008).

Makynen et al. (2000) reported an increase in E_2 level in fathead minnow exposed to 700 VZ μg/L. However, other studies do not show any estrogenic activity of VZ because of no changes in E_2 levels in fish exposed to VZ which are associated with no change in mRNA level of estrogenic biomarkers (Villeneuve et al., 2007; Martinovic et al., 2008). Taken together, these studies show that VZ potency to interfere with androgen production is uncertain and its estrogenic activity is unclear. In mammals, it has shown that VZ acts as an anti-androgen through androgen receptor pathway; however anti-androgenic activity of VZ on fish reproduction is poorly studied.

In the present study, we examined initially VZ-related brain-pituitary-testis dysfunctions leading to changes in E_2/11-KT balance in goldfish following acute and chronic exposures to VZ. We measured total cholesterol and high and low density lipoproteins (HDL and LDL) to clarify if disruption in 11-KT biosynthesis corresponds to substrate availability. For better understanding of VZ action, mRNA of genes encoding salmon gonadotropin releasing hormone (*gnrh3*), luteinising hormone receptor (*lhr*), follicle-stimulating hormone receptor (*fshr*), and selected genes encoding enzymes in the steroidogenesis pathway were investigated in the brain and testis. We have also studied changes of mRNA of the gene encoding *ar* in the brain and testis.

In another study, we established a combination protocol to better understand the effects of VZ on reproductive endocrine system where adult male goldfish were exposed to VZ, flutamide (Flu), testosterone (T) and their combinations for 10 d. Considering anti-androgenic activity of Flu and androgenic activity of T, this study provides new information to better understand whether VZ acts as an anti-androgen to disrupt fish reproduction. Neuroendocrine regulators of reproduction such as *kiss-1*, *kiss-2*, *gpr54*, *gnrh3*, and *ar* mRNA levels in the mid-brain and testicular *lhr* were studied.

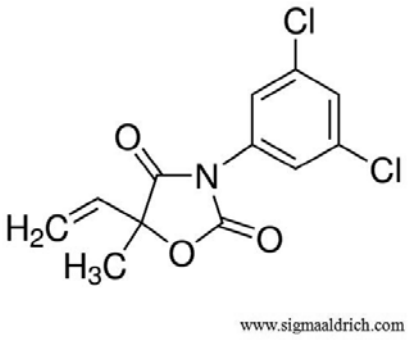

Figure 3. *Chemical structure of Vinclozolin.*

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

Species	Concentration (µg/L)	Stage	Endpoints	Authors
Zebrafish	10	Embryonic stage	Up regulation of *ar* mRNA	Smolinsky et al., 2010
Fathead minnow	100, 400 and 700	Maturity stage	No mortality Increase GSI at 400 and 700 No change in testicular development Increase in 11-KT at 400 and 700 No change in T Up regulation of *ar* mRNA at 700	Martinovic et al., 2008
Fathead minnow	100, 400 and 700	Maturity stage	Up regulation of *fsh*β mRNA at 700 No change in *lh*β Up regulation of *lhr* mRNA at 100, 400 and 700 no change in *fshr*	Villeneuve et al., 2007
Medaka	2500	From 1 to 100 dph	Inhibition in spermatogenesis Decrease in sperm density No intersex	Kiparissis et al., 2003
Guppy*	0.1, 1 and 10	Maturity stage	Decrease in sperm count at 1 and 10	Bayley et al., 2003
Guppy*	1, 10 and 100	Maturity stage	No change in testicular development	Kinnberg and Toft, 2003
Fathead minnow	75–1200	Embryonic stage	No change in sex ratio No change in fecundity	Makynen et al., 2000
Fathead minnow	200 and 700	Maturity stage	No change in GSI No change in testicular development No change in T and 11-KT Increase in E_2 at 700 No binding affinity to T in brain up to 50 µM	Makynen et al., 2000
Zebrafish	1000	Maturity stage	Up regulation of *fshr* Modulation of *spnr*, *hrb*, *dazl*	Martinovic et al., 2011

* Vinclozlin has been administrated by food (µg/mg food).
dpf, day post fertilization.
Gonadosomatic index, GSI; hepatosomatic index, HSI; vitellogenin, Vtg; Testosterone, T; 11-ketotestosterone, 11-KT; 17β estradiol, E_2; Luteinising hormone β, *lh*β; androgen receptor, *ar*; LH receptor, *lhr*; FSH receptor, *fshr*; spermatid perinuclear RNA-binding protein, *spnr*; HIV-1 Rev binding protein, *hrb*; deleted in azoospermia-like, *dazl*.
Zebrafish, *Danio rerio*; Fathead minnow, *Pimephales promelas*; Medaka, *Oryzias latipes*; Guppy, *Poecilia reticulate*.

5.2. Di-(2-ethylhexyl)-phthalate

Di-(2-ethylhexil) phthalate (DEHP) (Figure 4) is among the most used plasticizers in the plastic industries used as plasticizer in non-PVC materials, including natural and synthetic rubber, chlorinated rubber, polyvinyl butyral, nitrocellulose and ethyl cellulose. It has been detected in construction sector, packaging, transportation, wiring, medical devices, pharmaceuticals, paints, cosmetics and personal care products (Magdouli et al., 2013).

The Environmental Protection Agency has established a DEHP safety concentration limit in drinking water to be 6 μg/L (Clark et al., 2003). 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). Horn et al. (2004) measured 180, 62, and 4.6 μg/L in the St. Lawrence River water, Landfill leachate located inside the boundaries of the city of Montreal and tap water from Montreal water distribution system, respectively. The concentrations of DEHP reported 2.38 μg/L in the Southeast Queensland, Australia, 8.5–12.1 μg/L in USA, 41 μg/L in Canada, 6.85-122 μg/L in European countries (Magdouli et al., 2013).

It has shown that DEHP is capable of disturbing the reproductive success by interfering steroid hormones action as well as sperm production in mammal (Akingbemi et al., 2004; Latini et al., 2004; Andrade et al., 2006; Foster, 2006; Lee et al., 2009), while there is a significant lack of information about its effects on the reproductive performance of fish. It has been observed that DEHP decreases fertilization rate when male zebrafish are injected with 5000 mg/kg b.w. DEHP for 10 d (Uren-Webster et al., 2010) or exposed to 0.2–20 μg/L DEHP for 21 days (Corradetti et al., 2013). Male fertility also decreases in marine medaka exposed to 100 and 500 μg/L DEHP from hatching to maturity stage (Ye et al., 2014). However, it is still unknown whether DEHP-reduced male fertility is corresponding to DEHP effects on sperm quality factors, which are key determinants for male fertility in fish (Billard et al., 1995). Thibaut and Porte (2004) reported that DEHP inhibits 5 α-dihydrotestosterone syntheses in fish *in vitro*; a potent androgen that binds to androgen receptor (AR). However, *in vivo* studies show no change in T levels in mature fathead minnow (Crago and Klaper, 2012) and zebrafish (Uren-Webster et al., 2010) exposed to DEHP associated with no changes in *star* mRNA levels that encodes the steroidogenic acute regulatory protein to control the transfer of cholesterol into the steroidogenesis (Stocco, 2001). Ye et al. (2014) also observed no changes in T levels in marine medaka exposed to DEHP, but *star* mRNA levels were increased. In contrast, Wang et al. (2013) observed the increase in T levels in rare minnow exposed to DEHP. Overall, inhibitory effects of DEHP on androgen production are largely unknown in fish and none of the former studies have investigated 11-KT levels, which is the major androgen in spermatogenesis (Miura and Miura, 2003) (Table 2). In mammals, in prepubertal and *in utero* DEHP exposure decreases T levels at 10–750 mg/kg/d in male rats which was associated with changes in circulatory luteinizing hormone (LH) levels and *star* mRNA levels (Parks et al., 2000; Akingbemi et al., 2001, 2004; Ge et al., 2007; Howdeshell et al., 2007; Culty et al., 2008; Lee et al., 2009; Hannas et al., 2012). Similar to mammals (Akingbemi et al., 2004; Andrade et al., 2006), it has shown that DEHP also stimulates vitellogenin (Vtg) production or *vtg* mRNA in zebrafish (Uren-Webster et al., 2010), rare minnow (Wang et al., 2013) and marine medaka (Ye et al., 2014), associated with increases in 17 β-estradiol (E_2) and mRNA levels of genes encoding estrogen receptor subtypes (*er*). In contrast, Crago and Klaper (2012) reported decreases in E_2 levels and no change in mRNA levels of *er* subtypes and liver *vtg* mRNA in fathead minnow exposed to DEHP. Thus, DEHP estrogenic activity is still unclear and may differ among fish species.

In the present study, adult male goldfish were exposed to the range of DEHP doses from environmental relevant concentration to hotspot concentrations (1, 10, and 100 μg/L

DEHP) for a period of 30 days to study its effects on sperm quality factors and reproductive hormones. *star* mRNA levels were analysed to understand whether changes in sex steroid levels correspond to transfer of cholesterol into the steroidogenesis. To understand DEHP effects on neuroendocrine regulation of testicular hormonal functions, we analysed mRNA levels of *lhr*, salmon gonadotropin releasing hormone (*gnrh3*), and kisspeptin (*kiss1*) and its receptor (*gpr54*). Finally, estrogenic activity of DEHP was compared with E_2 treated goldfish by analyses of Vtg expression and the liver *vtg* mRNA levels as well as the mRNA levels of genes encoding brain P450 aromatase (*cyp19a1b*) and liver *erα*.

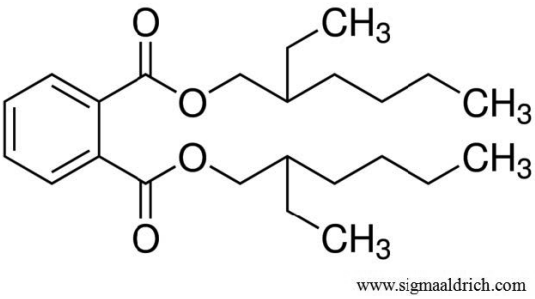

Figure 4. *Chemical structure of Di-(2-ethylhexyl)-phthalate.*

Table 2. *Summary of studies performed to investigate the effects of Di-(2-ethylhexyl)-phthalate on reproductive physiology in fish.*

Species	Concentration (µg/L)	Stage	Endpoints	Authors
Medaka	0.01, 0.1, 1, 10	Embryonic stage	Reduction in GSI Delay in hatching time No effect on hatching rate Distortion of sex ratio at low dose	Chikae et al., 2004
Marine medaka	100, 500	Embryonic stage	Increase in E_2 level No change in T Decrease in steroid ratios Increase Vtg level Up regulation of *erα, erβ, erɣ* at 0.1, brain No change in *gnrh2* and *arα* in brain Down regulation of *cyp19b* *ldlr, star, cyp17a1, 17βhsd, cyp19a* Reduce spermatozoa in tests	Ye et al., 2014
Zebrafish*	0.5, 50, 5000	Maturity stage	Increase HSI at 5000 Increase in the size and number of liver peroxisomes Decrease in the proportion of spermatozoa Increase in the proportion of spermatocytes Inhibit the progression of meiosis Up regulation of *vtg* in liver Up regulation of of *ppar, acox1, ehhadh*	Uren-Webster et al., 2010
Fathead minnow	12	Maturity stage	No change GSI No change in T Decrease in E_2 Decrease steroid ratio	Crago and Klaper, 2012
Atlantic salmon*	400, 800, 1500	Embryonic stage	Intersex fish (ovo-testis) at $1500*10^3$	Norman et al., 2005
Sticklebacks	15, 35	Maturity stage	Increase T at 35 No change in 11-KT Decrease in spiggine	Aoki et al., 2010
Medaka	1, 10, 50	From 1 to 90 dph	No effect on Male	Kim et al., 2002

Table 2. *Continued.*

Species	Concentration (µg/L)	Stage	Endpoints	Authors
Rare minnow	3.6, 12.8, 39.4, 117.6	Maturity stage	Increase in T Increase E_2 Increase level of Vtg Up regulation of *cyp17* and *cyp19* Down regulation of *erβ* in testis, brain at high doses Down regulation of *erα* and *erβ* and *cyp19b* in brain Up regulation of *erβ, erα* and *vtg* in liver at higher doses	Wang et al., 2013

dpf, day post fertilization.
* DEHP has been administrated by food (µg/mg food).
Gonadosomatic index, GSI; vitellogenin, Vtg; Testosterone, T; 11-ketotestosterone, 11-KT; 17β estradiol, E_2; estrogen receptor, *er*; LH receptor, *lhr*; FSH receptor, *fshr*; Peroxisome proliferator-activated receptor, *ppar*; aromatase gene, *cyp*; acyl-CoA oxidase 1, *acox1*.
Zebrafish, *Danio rerio*; Fathead minnow, *Pimephales promelas*; Medaka, *Oryzias latipes*; Guppy, *Poecilia reticulate;* Prussian carp, *Carassius gibelio*; Sticklebacks, Gasterosteidae; Atlantic salmon, *Salmo salar*; Marine medaka, *Oryzias melastigma.*

6. Aims of the present thesis

The main aim of present thesis were to study VZ and DEHP effects on neuroendocrines and endocrine regulation of reproduction in mature male goldfish following an acute and chronic exposure, *in vivo*. In particular, the following specific objectives were considered:

(a) Effects of VZ and DEHP on hypothalamus – pituitary – testis functions.
(b) Estrogenic activity of VZ and DEHP.
(c) The modes of action of VZ and DEHP through receptor pathway.

The present thesis provides new information to understand mechanisms by which VZ and DEHP disrupt neuroendocrine and endocrine regulation of reproduction in goldfish following an acute and chronic exposure.

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CHAPTER 2

REPRODUCTIVE ENDOCRINE SYSTEM IN GOLDFISH EXPOSED TO VINCLOZOLIN, *IN VIVO*

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Alternations in neuroendocrine and endocrine regulation of reproduction in male goldfish (*Carassius auratus*) following an acute and chronic exposure to vinclozolin, *in vivo*

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ABSTRACT

The fungicide vinclozolin (VZ) is in use globally and known to disrupt reproductive function in male. The present study tested the hypothesis that VZ disrupts testicular function in goldfish (*Carassius auratus*) by affecting brain–pituitary–testis axis. Goldfish were exposed to 100, 400 and 800 μg/L VZ and 5 μg/L 17β-estradiol (E_2) for comparison. In VZ treated goldfish, 11-ketotesteosterone (11-KT) secretion was changed depending on dose and duration period of treatment. Following 7 days of exposure, 11-KT was decreased in goldfish exposed to 800 μg/L VZ, while it was increased in goldfish exposed to 100 μg/L VZ after 30 days of exposure. Circulating E_2 level was unchanged in VZ treated goldfish, however the E_2/11-KT ratio was increased in a concentration-related manner. In E_2 treated goldfish, circulatory 11-KT and E_2 levels were decreased and increased, respectively, which resulted in an increase in the E_2/11-KT ratio. Exposure to VZ at 100 μg/L caused a significant increase in the circulatory luteinizing hormone (LH) after 30 days. In E_2 treated fish circulatory LH was decreased, significantly. Transcripts of genes encoding gonadotropin-releasing hormone and androgen receptor in the brain, and those of genes encoding LH and follicle-stimulating hormone receptors, StAR, CYP17, and 3β-HSD in the testis changed in VZ-treated goldfish depending on concentration and period of treatment. mRNA of genes encoding vitellogenin and estrogen receptor in the liver and cytochrome P450 aromatase in the brain were increased in E_2-treated goldfish. The results suggest that VZ-induced changes in 11-KT were due to disruption in brain–pituitary–testis axis and provide integrated characterization of VZ-related reproductive disorders in male fish.

1. Introduction

A large number of natural and synthetic compounds are known to be endocrine disrupting chemicals (EDCs) and interfere with metabolism and hormone synthesis and action resulting in adverse developmental and reproductive effects (Bergman et al., 2013; Jordan et al., 2012). In terms of reproductive disorders in males, EDCs have been linked to a decrease in semen quality (20–40% of young men in Europe) and an increase in global rates of testicular cancers (Andersson et al., 2008; Bergman et al., 2013). One of the findings is that EDCs with anti-androgenic properties (such as pharmaceuticals or pesticides) may alter androgen biosynthesis and subsequently cause severe impairment in male fertility (Andersen et al., 2002; Kojima et al., 2004; Hatef et al., 2013). In addition, anti-androgenic EDCs may affect secondary sexual characteristics in male amphibian and fish. However, EDC-related reproductive behavior alternations are not necessarily associated with male infertility (Bayley et al., 2003; Hoffmann and Kloas, 2010). There

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are gaps in our understanding of the mechanisms by which EDCs with anti-androgenic ability cause reproductive disorders in vertebrates. This is in part due to limited information available on the role of androgen receptors (AR) in androgen-mediated testicular development and functions (Borg, 1994; Luccio-Camelo and Prins, 2011).

Vinclozolin (VZ) is a dicarboximide fungicide widely used for control of diseases in grapes, fruits, and vegetables (Pothuluri et al., 2000). It has been listed under the European Union priority list of EDCs (European Commission, 2001) and in the United States Environmental Protection Agency's final Contaminant Candidate List 3 for products that should be assessed for contamination levels in drinking water (U.S. EPA, 2009). People may be exposed to residues of VZ through food or drinking water. In surface waters, VZ has been detected at concentrations of 0.1–2.4 μg/L (Readman et al., 1997; El-Shahat et al., 2003).

It has been reported that VZ causes disruption in sexual differentiation, testicular development and sex steroid biosynthesis leading to abnormal sperm in mammals (Gray et al., 1994, 1999; Kubota et al., 2003; Veeramachaneni et al., 2006; Loutchanwoot et al., 2008; Eustache et al., 2009; Auger et al., 2010), amphibians (Kloas, 2002; Hoffmann and Kloas, 2010) and fish (Baatrup and Junge, 2001; Bayley et al., 2003; Kiparissis et al., 2003; Hatef et al., 2012). Surprisingly, reproductive impairments caused by VZ could be transgenerationally transmitted by an epigenetic mechanism (Anway et al., 2005). The present study aimed at providing more integrated characterization of VZ effects on neuroendocrine and endocrine regulation of reproduction in fish as a model organism.

Previous studies showed no change in circulating testosterone (T) or 11-ketotestosterone (11-KT) levels following exposure of fathead minnow to VZ, *in vivo* (Makynen et al., 2000; Martinović et al., 2008). Despite of the lack of change in androgen level in the blood plasma, the same authors reported an increase in mRNAs of genes which encode AR, luteinizing hormone receptor (LHr) and 11β-HSD (an enzyme which converts T to 11-KT), while mRNA of the gene encoding LH remained unchanged (Villeneuve et al., 2007; Martinović et al., 2008). In contrast, we have recently observed a concentration-dependent 11-KT biosynthesis in goldfish exposed to VZ (Hatef et al., 2012). In the present study, our main objective was to examine VZ-related brain–pituitary–testis dysfunctions leading to changes in 11-KT biosynthesis. We have studied 11-KT and E_2/11-KT balance (a key factor for normal testicular development, Quignot et al., 2012) in goldfish following acute and chronic exposures to VZ. We measured total cholesterol and high and low density lipoproteins (HDL and LDL) to clarify if disruption in 11-KT biosynthesis corresponds to substrate availability. For better understanding of VZ action, mRNAs of genes encoding salmon gonadotropin releasing hormone (GnRH3), LHr, follicle-stimulating hormone receptor (FSHr), and selected genes encoding enzymes in the steroidogenesis pathway were investigated in the brain and testis. We have also studied changes of mRNA of the gene encoding AR in the brain and testis. It has been demonstrated that VZ acts through an androgen receptor (AR) antagonist mode of action in mammals (Kelce et al., 1994; Wong et al., 1995), but VZ binding affinity to AR is uncertain in fish (Makynen et al., 2000). Overall, the present study provides new information to understand mechanisms by which VZ disrupts brain–pituitary–testis functions in fish reproduction following an acute and chronic exposure.

2. Materials and methods

2.1. Exposure to vinclozolin: experimental design and sampling

In this study, we used samples of mature male goldfish (2–3 years old), which were exposed to VZ. Results on the effects of VZ on sperm quality and sex steroid following 30 days exposure have been already published (see Hatef et al., 2012). Briefly, goldfish were exposed to 100, 400, and 800 μg/L VZ (nominal concentrations), one estrogenic compound (E_2, 5 μg/L) and one solvent control (acetone). All groups were held under same conditions: the aquaria (each 80 L), temperatures (18–22 °C, at the beginning and end of experiment, respectively) and photoperiod (12 h light/12 h dark). Dissolved oxygen level and pH of 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). Ten aquaria were used (2 per each group) and 3 individuals were sampled from each aquarium (n = 6 per each group). Every 48 h, total volume of water was renewed. Before sampling, individuals were anaesthetized in a 2-phenoxyethanol solution (0.3 ml/L). Samples of blood (about 1 mL from each specimen) were taken from the caudal vein and collected at day 7, 15 or 30 of exposure using a heparinized syringe. Plasma was collected following centrifugation at 5000 rpm for 10 min at 4 °C and stored at −80 °C until use. Samples of testis, liver and brain were collected at day 7 and 30 following exposure and were immediately frozen in liquid nitrogen and kept in an RNAse free tube at −80 °C until use. There were no between-group differences in fish weight and length (Hatef et al., 2012). All animals were handled according to §17 odst. 1 zakona No. 246/119 Sb, Ministry of Agriculture, approved by Central Ethics Committee of the Ministry of Health of the Czech Republic.

2.2. Sex steroid measurements

Actual concentrations of 11-KT and E_2 and the E_2/11-KT ratio were assessed in blood plasma following 7, 15 and 30 days exposure according to Hatef et al. (2012). Blood plasma was diluted in a specific buffer provided by the Cayman EIA kit (Cayman, Michigan, USA) or by the Diasource EIA kit (Diasource, Nivelles, Belgium) for 11-KT and E_2 assays, respectively. Fifty μL of each control, 11-KT or E_2 standard and blood plasma were dispensed in separate wells. Antibody against 11-KT or E_2 and tracer (each 50 μL) were added into each well and incubated for 2 h at room temperature (RT). Following washing, adding the substrate solution (200 μL) and incubation of plates for 30–90 min at RT, the reaction was the stopped by adding 100 μL of H_2SO_4. The optical density was read for 11-KT and E_2 at 405 and 450 nm, respectively, with a microtiter plate reader. For E_2, sensitivity was 5 pg/mL, and intra-assay and inter-assay coefficients of variation were 4% and 6%, respectively, for a plasma concentration in the range of 100–250 pg/mL. For 11-KT, 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 values was used.

2.3. Luteinizing hormone (LH) assay

Circulating LH level was assayed in the blood plasma by an ELISA method, previously established for LH assay in common carp (Kah et al., 1989). To validate the assay, serial dilutions of blood plasma were made together with a common carp standard. All were found to be parallel with the sensitivity of the assay in the range 0.6–100 ng ml^{-1} and the intra- and inter-assay coefficients of variance were 5% and 9%, respectively.

2.4. Cholesterol measurements

Cholesterol (CHOD-PAP) reagent was used for the determination of total cholesterol, HDL and LDL in the blood plasma using an enzymatic colorimetric method following the manufacturer's instructions (NS Biotec, Dunn Labortechnik GmbH, Asbach, Germany). Following the preparation of samples, the intensity of the color produced at 500–550 nm (total cholesterol) is

directly proportional to cholesterol concentration, HDL or LDL concentrations were determined by measuring the absorbance at 530–570 nm.

2.5. RNA extraction and complementary DNA synthesis (cDNA)

Total RNA was extracted using a PureLink RNA Mini Kit (Cat. No. 12183018A, 12183025) and removing DNA *via* on-column PureLink DNase treatment (Cat. No. 12185-010) following the manufacturer's instructions (Invitrogen). Total RNA concentration was estimated from absorbance at 260 nm (A260 nm, NanoDrop, USA) and its quality was verified by A260 nm/A280 nm ratios between 1.8 and 2 and A230 nm/A260 nm ratios greater than 2. cDNA was synthesized from 2 μg total RNA of each sample using a high capacity cDNA reverse transcription kit with RNase Inhibitor (Applied Biosystems, Cat. No. 4374966) following the manufacturer's instructions. Briefly, 2 μg total RNA of each sample was added to nuclease free water to reach 10 μL. Then, 2 μL of 10× RT buffer, 0.8 μL of 25× dNTP Mix (100 mM), 2 μL of 10× RT Random Primers, 1 μL of multiscribe reverse transcriptase, 1 μL of RNase inhibitor and 3.2 μL of nuclease free water were added. The reaction mixture was then incubated at 25 °C for 10 min and at 37 °C for 120 min using an iQ cycler. By heating at 85 °C for 5 min, the reaction was deactivated. A negative control was run for each sample. Each 20 μL reaction was diluted three-fold in nuclease-free water and used as a template for quantitative real-time polymerase chain reaction (qRT-PCR) assay.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

The iCycler iQ Real-time PCR Detection System (Bio-Rad Laboratories) was used for evaluating gene expression (in this article, the phrase gene expression is taken to be synonymous to gene transcription, but it is acknowledged that gene expression can also be regulated, *e.g.*, by translation and protein stability) level with the following condition per reaction: 1 mL diluted cDNA, 0.26 mM each primer, 12.5 mL SYBR Green PCR Master Mix (Qiagen Mississauga, Canada), and ultrapure distilled water (Invitrogen) to a total volume of 25 mL. Specific primers designed for each gene and housekeeping genes are shown in Table 1. The PCR was run in the following cycling conditions: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C, appropriate annealing temperatures for 31 s. The specificity of the amplified product in the qRT-PCR assay was determined by analyzing the melting curve to discriminate the target amplicon from the primer dimer or other nonspecific products. A single melt curve was observed for each primer set in all qRT-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 levels were normalized to those of GAPDH or β-actin mRNA using the standard $2^{-\Delta\Delta Ct}$ method.

2.7. Statistical analyses

Homogeneity of variance and normal distribution of data were tested using the Levene's test and Kolmogorov–Smirnov's test. As needed, data were transformed to meet assumptions of normality and homoscedasticity. In this study, we examined the effects of VZ at each exposure time (acute or chronic), independently. Therefore, a single ANOVA following the Tukey–Kramer test was performed to understand the effects of VZ on each parameter in an acute and a chronic exposure. Alpha = 0.05 was set to indicate significant differences at each sampling time. In this context data from VZ-treated groups were compared with the solvent control and between VZ-treated groups. Similarly, the E_2-treated group was compared with the solvent control and with VZ-treated groups. All data are presented as mean ± standard error of mean (SEM).

3. Results

3.1. Sex steroid concentrations

At day 7, 11-KT level was decreased in goldfish exposed to 800 μg/L VZ compared to control ($p < 0.05$, Fig. 1). There was no difference in 11-KT level at day 15 following exposure to VZ (Fig. 1). At day 30 following exposure, a biphasic change in 11-KT was observed. The circulating level of 11-KT was increased at the lowest VZ concentration (100 μg/l), but it was decreased in goldfish exposed to 800 μg/L VZ ($p < 0.05$, Fig. 1). In the positive control group, exposure to E_2 decreased 11-KT concentration following 7, 15 or 30 days compared to control ($p < 0.05$, Fig. 1).

No difference in plasma E_2 level was observed in goldfish exposed to VZ (Fig. 1). As expected, exposure to E_2 led to increase in circulating E_2 level in goldfish at all time points tested ($p < 0.05$, Fig. 1).

In goldfish exposed to VZ, the E_2/11-KT ratio was increased in a concentration-dependent manner increasing significantly above control at 800 μg/L VZ at 7 days of exposure ($p < 0.05$, Fig. 1), but remained unchanged after 15 days of exposure (Fig. 1). After 30 days of exposure, however, 100 μg/L VZ decreased the E_2/11-KT ratio which was increased in goldfish exposed to 800 μg/L VZ ($p < 0.05$, Fig. 1).

Table 1
Primer sequences and annealing temperature applied for studying alternations in mRNA of selected genes using qRT-PCR in the present study.

Genes	Forward primer	Reverse primer	Annealing temperature (°C)
GnRH3	TGGTCCAGCTCCTAATGCTA	AACACTTCTCTTTCCACCCG	57
LHr	CCTCTGCATCGGTGTGTATC	TAGACAGATAGTTCGCCGCC	60
FSHr	CGTCCACAATCCTACCTTCG	TGAGAAACGGTGATTAGCGG	58
AR	GATGAAAGGTCCAGAGGAGG	ACTGTGAGTGGAACGTCAGG	57
StAR	ATGGCTGGCAAACTGAGATCGAGA	TCCATGTTATCCACCAGCTCCTCA	58
3β-HSD	GAAGAGCGTGTAAAGGAGCA	ACGTTTTCTTGGATGCAGGT	58
CYP17	GCACAAGGTGGATTACAGCG	GAAGATGTCCCCCACAGTCA	58
CYP19a1a	TTGTGCGGGTTTGGATCAATGGTG	TTCCGATACACTGCAGACCCAGTT	58
CYP19a1b	AGGCGAGCGGGATGTAGAGT	CGTCCGATGTTCAGGATGAGG	58
Vtg	GAAGTGCGCATGGTGGCTTGTATT	AGCTGCCATATCAGGAGCAGTGAT	55
ERα	GAGGAAGAGTAGCAGCACTG	GGCTGTGTTTCTGTCGTGAG	55
GAPDH	TGATGCTGGTGCCCTGTATGTAGT	TGTCCTGGTTGACTCCCATCACAA	57
β-actin	GTTTTGCTGGAGATGATGCC	TTCTGTCCCATGCCAACCAT	58

Genes encoding salmon gonadotropin releasing hormone (*GnRH3*), luteinizing hormone receptor (*LHr*), follicle-stimulating hormone receptor (*FSHr*), androgen receptor (*AR*), steroidogenic acute regulatory protein (*StAR*), 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase (*3β-HSD*), cytochrome P450 17 (*CYP17*), testicular aromatase (*CYP19a1a*), brain aromatase (*CYP19a1b*), vitellogenin (*Vtg*), estrogen receptor subunit α (*ERα*). The gene encoding glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as house keeping gene in the liver and testis. The gene encoding β-actin was used for mRNA analysis of genes in the brain.

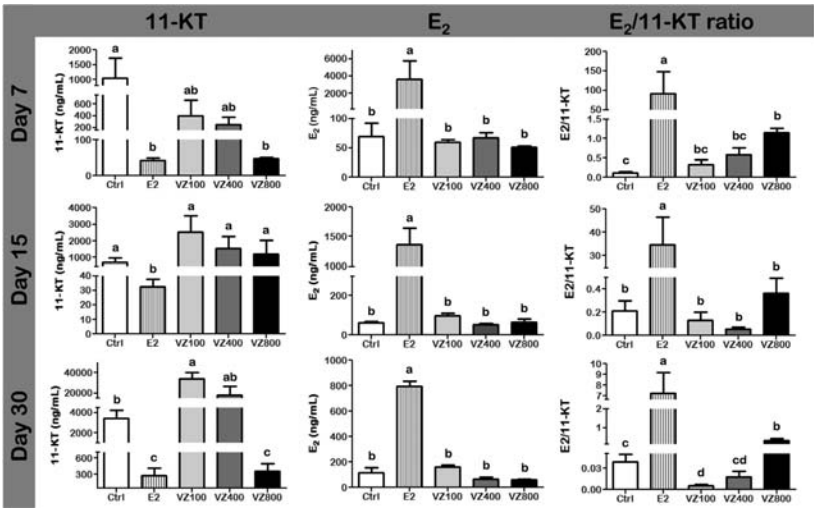

Fig. 1. 11-ketotestosterone (11-KT), 17β-estradiol (E_2), and E_2/11-KT ratio in the blood plasma of male goldfish exposed to vinclozolin (nominal concentrations 100, 400 or 800 μg/L VZ) and to 17β-estradiol (E_2), *in vivo*. Mature goldfish were exposed to VZ or E_2 and blood samples were collected for analysis of 11-KT, E_2 and E_2/11-KT ratio at 7, 15 and 30 days of exposure. Data is expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's post hoc test).

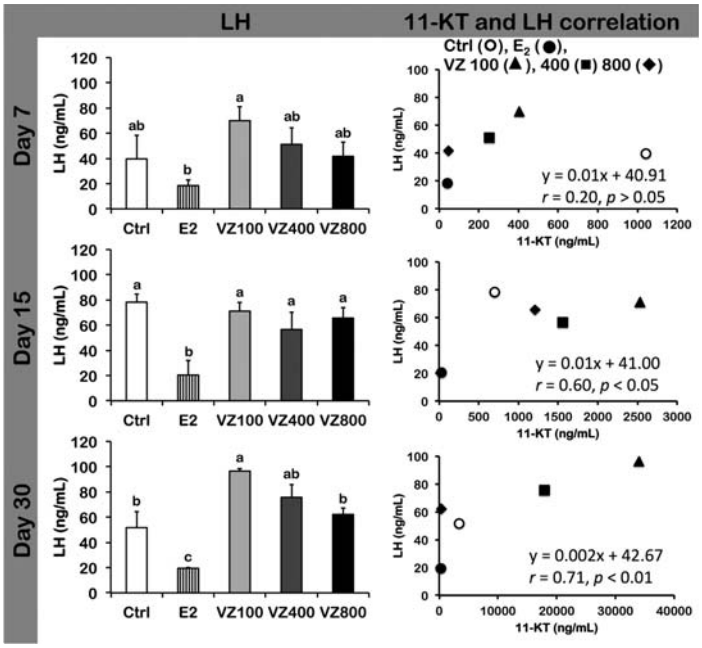

Fig. 2. Circulating luteinizing hormone (LH) level and correlation between LH and 11-ketotestosterone (11-KT) concentrations in the blood plasma of male goldfish exposed to vinclozolin (nominal concentrations 100, 400 or 800 μg/L VZ) and to 17β-estradiol (E_2), *in vivo*. Mature goldfish were exposed to VZ or E_2 and blood samples were collected for analysis of LH following 7, 15 and 30 days of exposure. For LH, data is expressed as mean ± SEM ($n = 6$) and values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's post hoc test). For studying correlation between LH and 11-KT, means of LH and 11-KT levels from each group were applied.

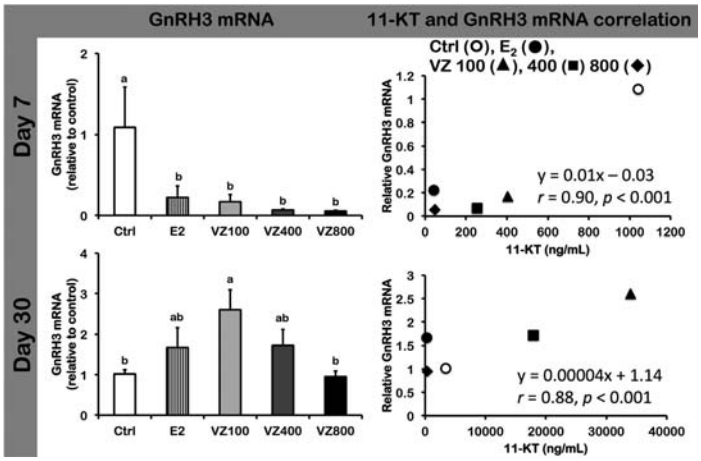

Fig. 3. mRNA of the gene encoding brain salmon gonadotropin-releasing hormone (*GnRH3*) mRNA and correlation between brain *GnRH3* mRNA and blood plasma 11-ketotestosterone (11-KT) concentrations in male goldfish exposed to vinclozolin (nominal concentration 100, 400 or 800 μg/L VZ) and to 17β-estradiol (E_2), *in vivo*. Mature goldfish were exposed to VZ or E_2 and samples of brain were collected for studying alternations in *GnRH3* mRNA using a quantitative real-time PCR following 7 and 30 days of exposure. Data were normalized to the internal control and is expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's post *hoc* test). For studying correlation between LH and 11-KT, means of LH and 11-KT levels from each group were applied.

3.2. Luteinizing hormone (LH) and mRNA of the gene encoding salmon gonadotropin-releasing hormone (GnRH3)

Following 7 and 15 days of exposure, circulating LH level was unchanged in goldfish exposed to VZ compared to control (Fig. 2). A significant increase in circulating LH was observed in goldfish exposed to 100 μg/L VZ for 30 days ($p < 0.05$, Fig. 2); higher concentrations were without effect. In the positive control group exposed to E_2, circulating LH was decreased after 7, 15 or 30 days compared to control ($p < 0.05$, Fig. 2). When the mean values of circulating LH and 11-KT for each group were considered for regression analysis, positive correlations were observed between them following 15 and 30 days of exposure (Fig. 2). It is worth to pointing out that circulating LH level in the control group was higher at day 15 of the experiment compared to those of days 7 and 30, suggesting that goldfish were at the spermiation stage.

As in E_2 treated goldfish, exposure of goldfish to all VZ concentrations decreased *GnRH3* mRNA level in the brain after 7 days compared to the control ($p < 0.05$, Fig. 3). Exposure to 100 μg/L VZ for 30 days significantly increased the brain *GnRH3* mRNA level ($p < 0.05$) without exerting the effect at higher VZ concentrations (400–800 μg/L) (Fig. 3). Nor did exposure to E_2 have any effect on *GnRH3* mRNA level at 30 days following exposure ($p < 0.05$, Fig. 3). 11-KT biosynthesis was associated with GnRH3 and LH synthesis and secretion in VZ-treated goldfish. When the mean values of GnRH3 and 11-KT for each group were considered for regression analysis, positive correlations were observed between them following 7 and 30 days of exposure (Fig. 3).

3.3. mRNAs of genes encoding receptors of luteinizing hormone (LHr) and follicle-stimulating hormone (FSHr)

At 7 days following exposure, *LHr* mRNA was increased in the testis of goldfish exposed to 400 and 800 μg/L VZ compared to the control ($p < 0.05$, Fig. 4A). A significant increase in testicular *FSHr*

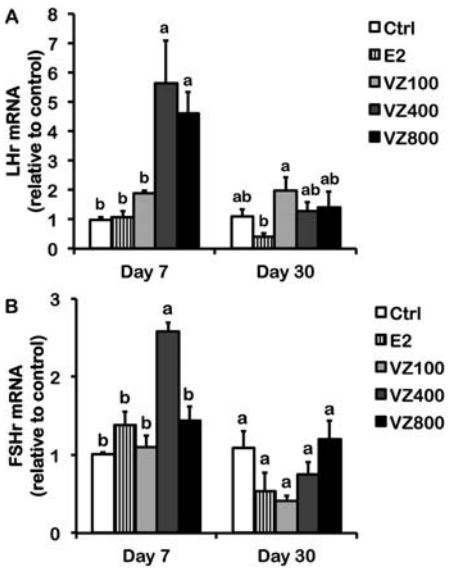

Fig. 4. mRNAs of genes encoding luteinizing hormone receptor (*LHr*) and follicle-stimulating hormone receptor (*FSHr*) in the testis of male goldfish exposed to vinclozolin (nominal concentrations 100, 400 or 800 μg/L VZ) and to 17β-estradiol (E_2), *in vivo*. Mature goldfish were exposed to VZ or E_2 and samples of testis were collected for studying alternations in *LHr* mRNA (A) and *FSHr* mRNA (B) using quantitative real-time PCR. Data were normalized to the internal control and is expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's post hoc test).

Table 2
Plasma cholesterol, low-density lipoprotein and high-density lipoprotein levels (mg/dL) in male goldfish (*Carassius auratus*) exposed to vinclozolin (VZ, 100, 400 and 800 μg/L) for one month. Samples of blood plasma were collected at day 7, 15 and 30 following exposure. Data are expressed as mean ± SEM ($n = 6$). For each parameter, there are no treatment differences at the same sampling time ($p > 0.05$).

Parameter	Groups	Period of exposure		
		7 days	15 days	30 days
Cholesterol	Ctrl	90.98 ± 19.84	94.75 ± 7.62	93.95 ± 10.09
	VZ100	115.48 ± 8.35	119.85 ± 11.75	120.92 ± 7.03
	VZ400	102.58 ± 12.66	90.10 ± 10.38	124.97 ± 19.11
	VZ800	101.57 ± 15.43	79.25 ± 17.31	88.48 ± 15.95
Low density lipoproteins	Ctrl	25.77 ± 7.29	26.28 ± 2.11	31.98 ± 3.38
	VZ100	34.03 ± 4.97	34.15 ± 3.61	40.15 ± 2.80
	VZ400	27.18 ± 2.19	26.53 ± 3.64	43.25 ± 7.49
	VZ800	27.50 ± 5.23	24.70 ± 6.37	29.98 ± 6.64
High density lipoproteins	Ctrl	33.12 ± 5.00	35.00 ± 0.64	34.10 ± 4.65
	VZ100	45.52 ± 3.27	37.25 ± 3.56	41.25 ± 3.82
	VZ400	42.65 ± 4.79	34.10 ± 3.10	42.67 ± 6.33
	VZ800	36.47 ± 5.14	24.48 ± 5.87	25.28 ± 6.80

Regulatory gene encoding enzymes to produce androstenedione.

mRNA was also observed in goldfish exposed to 400 μg/L VZ for 7 days ($p < 0.05$, Fig. 4B). Exposure to E_2 was without effect on *LHr* and *FSHr* mRNA in the testis (Fig. 4A and B). Following 30 days, *LHr* and *FSHr* mRNA levels remained unchanged in the testis of goldfish exposed to VZ or to E_2 (Fig. 4A and B).

3.4. Total cholesterol, high and low density lipoproteins (HDL and LDL)

Total cholesterol, HDL and LDL levels remained unchanged in goldfish following exposure to 100, 400 or 800 μg/L VZ (Table 2). The results show that 11-KT biosynthesis in goldfish exposed to VZ could not be related to precursor availability in sex steroid biosynthesis. These parameters were not measured in the E_2-treated group, because blood samples were not available. However, a previous study shows no changes in total cholesterol, HDL and LDL levels in E_2 treated goldfish (Sharpe et al., 2007).

3.5. Selected steroidogenic enzyme genes in steroidogenesis pathway

Exposure to 100 and 400 μg/L VZ increased *StAR* mRNA level in the goldfish testis following 7 days of exposure ($p < 0.05$), while VZ at 800 μg/L was without effect (Fig. 5). After 30 days, VZ increased *StAR* mRNA level at 100 μg/L ($p < 0.05$), but was without effect at higher doses (Fig. 5). In E_2-treated goldfish, *StAR* mRNA was decreased following 7 days ($p < 0.05$) and remained unchanged after 30 days of exposure (Fig. 5).

The testicular mRNA level of the gene encoding cytochrome P450 17 (*CYP17*) increased following exposure of goldfish to 400 μg/L VZ for 7 days and to 100 and 400 μg/L for 30 days compared to the control ($p < 0.05$, Fig. 5). Exposure to E_2 was without effect on *CYP17* mRNA in the testis.

We also measured mRNA of the gene encoding 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase (*3β-HSD*) mRNA

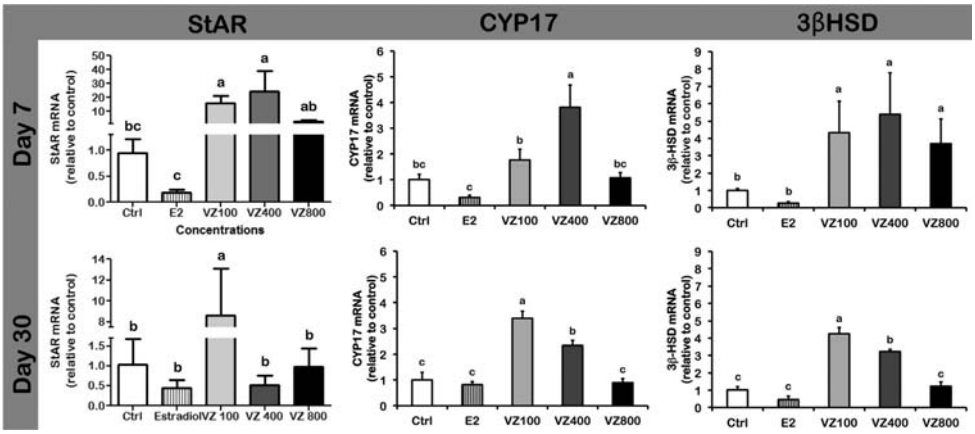

Fig. 5. mRNAs of genes encoding steroidogenic acute regulatory protein (*StAR*), cytochrome P450 17A1 (*CYP17*) and 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase (*3β-HSD*) mRNA in the testis of male goldfish exposed to vinclozolin (nominal concentration 100, 400 or 800 μg/L VZ) and to 17β-estradiol (E_2), *in vivo*. Mature goldfish were exposed to VZ or E_2 and samples of testis were collected for studying alternations in mRNA of genes following 7 and 30 days of exposure using quantitative real-time PCR. Data were normalized to the internal control and are expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's post hoc test).

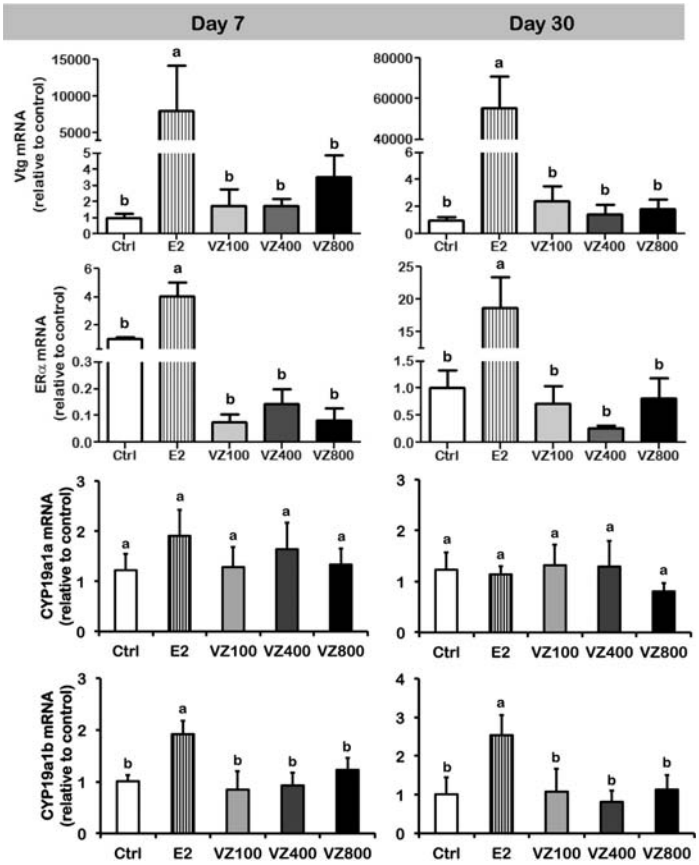

Fig. 6. Alternations in the mRNA of the genes encoding cytochrome P450 aromatase (*CYP19a1a*) in the testis, *CYP19a1b* in the brain and vitellogenin (*Vtg*) and estrogen receptor subunit α (*ERα*) in the liver of male goldfish exposed to vinclozolin (nominal concentration 100, 400 or 800 μg/L VZ) and to 17β-estradiol (E_2), *in vivo*. Mature goldfish were exposed to VZ or E_2 and samples of brain, testis or liver were collected for studying alternations in mRNA of the genes using quantitative real-time PCR following 7 and 30 days of exposure. Data were normalized to the internal control and are expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's post hoc test).

level in the goldfish testis. Exposure to VZ significantly increased *3β-HSD* mRNA level after 7 (100, 400 and 800 μg/L) and 30 (100 and 400 μg/L) days of exposure (Fig. 5). Exposure to E_2 was without effect on *3β-HSD* mRNA in the testis.

3.6. mRNAs of the genes encoding vitellogenin (Vtg), cytochrome P450 aromatase (CYP19a1a and CYP19a1b), and estrogen receptor subunit α (ERα)

There were no differences in brain *CYP19a1b* mRNA, testicular *CYP19a1a* mRNA and liver *Vtg* mRNA or *ERα* mRNA of goldfish exposed to any VZ concentration for either 7 or 30 days compared to the control ($p > 0.05$, Fig. 6). Exposure to E_2 increased brain *CYP19a1b* mRNA, liver *Vtg* and *ERα* mRNA levels after 7 or 30 days ($p < 0.05$), while testicular *CYP19a1a* mRNA remained unchanged compared to the control (Fig. 6). These results demonstrate that VZ did not have E_2-like activity.

3.7. mRNA of the gene encoding androgen receptor (AR) in the brain and testis

Exposure of goldfish to VZ (100, 400, 800 μg/L) or E_2 reduced *AR* mRNA level in the brain after 7 days compared to the control ($p < 0.05$, Fig. 7). After 30 days, *AR* mRNA level was increased in the brain of goldfish exposed to 100 μg/L VZ, while other VZ concentrations and E_2 were without effect ($p > 0.05$, Fig. 7).

In the testis, exposure to 400 μg/L VZ significantly increased *AR* mRNA level after 7 days of exposure ($p < 0.05$) and other treatments were without effect (Fig. 7). After 30 days, VZ was without effect on *AR* mRNA in the testis ($p > 0.05$, Fig. 7). Exposure to E_2 was without effect on testicular *AR* mRNA level ($p > 0.05$, Fig. 7).

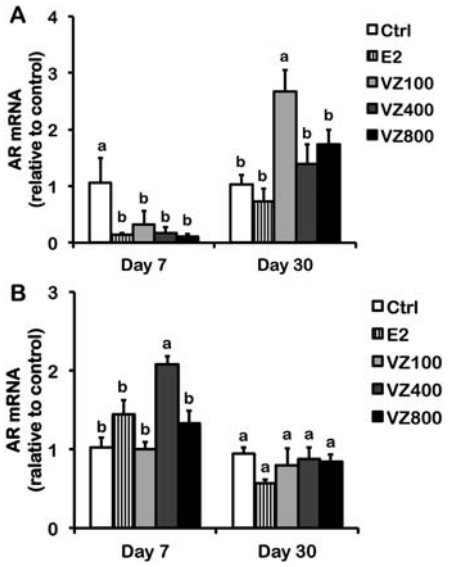

Fig. 7. mRNA of the gene encoding androgen receptor (*AR*) in the brain and testis of male goldfish exposed to vinclozolin (nominal concentration 100, 400 or 800 μg/L VZ) and to 17β-estradiol (E_2), *in vivo*. Mature goldfish were exposed to VZ or E_2 and samples of brain and testis were collected for studying alternations in *AR* mRNA using quantitative real-time PCR following 7 and 30 days of exposure. Data were normalized to the internal control and are expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's post hoc test).

4. Discussion

We previously observed that VZ decreases sperm quality in goldfish following a chronic exposure (30 days) at 800 μg/L, which was associated with a decrease in 11-KT level (Hatef et al., 2012). These suggest that VZ-induced endocrine changes may result in diminished sperm quality, a key determinant for male fertility in fish (Alavi et al., 2008; Hatef et al., 2013). For better understanding of VZ-related testicular dysfunctions, the present study was carried out to investigate the effects of VZ on steroid biosynthesis by analysing changes in *GnRH3*, *LHr*, *FSHr* and *AR* mRNA levels as well as studying the expression of a selected number of genes encoding enzymes in steroidogenic pathway. The results demonstrate that VZ-related reproductive disorders depend on the concentration and period of exposure (Fig. 8).

In the present study, we observed a biphasic effect of VZ on 11-KT production. A chronic exposure (30 days) to 100 μg/L VZ increased 11-KT production; while at the highest concentration studied (800 μg/L) it was decreased compared to the control. Responses to acute exposure (7 days) differed ranging from inhibition of 11-KT production at 800 μg/L to no effect at lower concentrations (100 and 400 μg/L). In mammals, VZ-induced increase in T production has been reported in male rats (Kubota et al., 2003; Loutchanwoot et al., 2008; Quignot et al., 2012), while in fish VZ causes no change in 11-KT production in fathead minnows exposed for 21 days (Makynen et al., 2000; Martinović et al., 2008). It should be noted that a similar range of concentrations of VZ were tested on fathead minnows (100–700 μg/L). In E_2 treated fish, the 11-KT level decreased following either acute or chronic exposure indicating that E_2 impairs androgen synthesis in fish (Yamaguchi et al., 2006; Sharpe et al., 2007). The results reveal similarity between E_2 and a high VZ concentration in inhibiting 11-KT production. In contrast, VZ induced 11-KT production at low concentration.

This is the first study that provides information on the effect of VZ on circulating LH levels in fish, which is the primary regulator of androgen biosynthesis in the testis (Nagahama, 1994). Following a chronic exposure for 30 days, LH level increased in goldfish exposed to 100 μg/L VZ, which is consistent with the observed increase in 11-KT level. This indicates that 11-KT elevation in goldfish exposed to 100 μg/L VZ was likely caused by increased production of LH from pituitary, suggesting VZ-induced disruption of the negative feedback of androgen to control LH release (Van der Kraak, 2009). Similarly, VZ-induced androgen production mediated by LH has also been observed in male rats (O'Connor et al., 2002; Kubota et al., 2003; Loutchanwoot et al., 2008). An earlier study on fathead minnows demonstrated no changes in *LH* mRNA level following exposure to VZ (Villeneuve et al., 2007). This is possible since *LH* mRNA levels may not always correspond with LH protein level as has been demonstrated previously in goldfish (Huggard-Nelson et al., 2002; Klausen et al., 2005; Moussavi et al., 2012, 2013). In E_2 treated goldfish, decrease of both circulating LH and 11-KT indicates E_2-related inhibition of 11-KT production mediated by LH release from the pituitary. Another contributing factor could be increase in plasma E_2 level, since there is evidences that E_2 regulates LH production through both positive and negative feedback directly or mediated by GnRH function (Huggard-Nelson et al., 2002; Van der Kraak, 2009).

In the present study, we also investigated upstream neuroendocrine regulator for LH production (Nagahama, 1994). Results showed that *GnRH3* mRNA was decreased in goldfish exposed to VZ after 7 days exposure, which is consistent with the observed decrease in 11-KT levels. The significant increase in *GnRH3* mRNA level following 30 days exposure is also consistent with the observed increase in 11-KT level in the same period following VZ exposure. This is first study that provides information on VZ-induced disruption in brain *GnRH3* expression and links this effect to pituitary LH production, which inhibits 11-KT biosynthesis. Interestingly, *GnRH3* mRNA in E_2 treated goldfish was unchanged following 30 days exposure, suggesting that inhibition of LH release might be mediated indirectly by E_2 and other neurohormones such as dopamine (Sokolowska et al., 1985; Trudeau et al., 1993).

We analyzed mRNA levels of selected steroidogenic enzyme genes to investigate whether VZ-induced neuroendocrine disruption may be associated with 11-KT biosynthesis in the testis. The *StAR* (a regulatory gene for cholesterol transfer within the mitochondria, Stocco, 2001) mRNA increased in goldfish exposed to 100–400 μg/L and 100 μg/L VZ following 7 and 30 days exposure, respectively. *CYP17* (a regulatory gene encoding enzymes to produce androstenedione) mRNA also increased in goldfish exposed to 100 μg/L VZ following 7 days and to 100–400 μg/L VZ following 30 days exposure. In addition, *3β-HSD* (a gene encoding the enzyme to convert pregnenolone to progesterone or to produce androstenedione from dehydroepiandrosterone) mRNA also increased in goldfish following 7 and 30 days exposure to VZ. The observed increases in *StAR*, *CYP17* and *3β-HSD* mRNAs following chronic exposure to 100 μg/L VZ were correlated with an increase in *GnRH3* mRNA, and circulating LH level, suggesting that VZ action is exerted at multiple levels and effects substrate availability for 11-KT biosynthesis. These results confirm a previous study in which the increase in 11-KT levels was associated with the increase in the mRNA of the gene encoding cytochrome P450 side chain cleavage (*P450scc*). The gene product catalyzes conversion of cholesterol to pregnenolone within the mitochondria (Kubota et al., 2003). What is interesting is that mRNA elevation in almost all of these

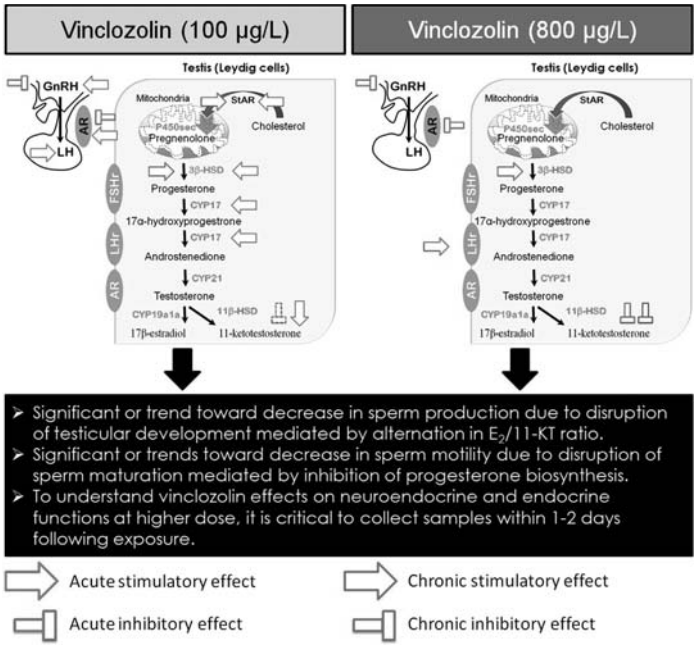

Fig. 8. A model representing neuroendocrine and endocrine disorders in goldfish leading to diminished sperm quality following an acute and chronic exposure. A chronic exposure to lower concentration (100 µg/L) of vinclozolin (VZ) increased 11-KT level due to an increase in *GnRH3* mRNA and circulating LH level associated with an increase in mRNA of genes encoding steroidogenic enzymes in the testis and AR in the brain. An acute exposure of goldfish to 100 µg/L VZ showed a trend toward decrease in 11-KT associated with a decrease in *GnRH3* and *AR* mRNA. However, mRNAs of genes encoding steroidogenic enzymes was increased. At higher concentration (800 µg/L), 11-KT level was decreased following an acute and chronic exposure, while circulating LH level and mRNAs of genes encoding steroidogenic enzymes remained unchanged. Decrease in 11-KT was associated with decrease in *GnRH3* mRNA and brain *AR* mRNA following an acute exposure.

genes happened earlier than VZ effects on 11-KT level observed at 30 days following exposure. This suggests that VZ may affect testis function directly and more quickly than the additional disruption caused to neuroendocrine function. It may be important to investigate changes in mRNA of genes encoding steroidogenic enzymes within 1–2 days following exposure to VZ for better understanding of its effects on 11-KT biosynthesis following an acute exposure. In this regard, it has been shown that ketoconazole (an anti-androgenic EDCs) displays time-course effects to disrupt steroidogenesis resulting in alternations in circulating steroid levels in fish (Ankley et al., 2012). In E_2-treated fish, mRNAs of steroidogenic enzyme genes decreased following both acute and chronic exposure, suggesting that the decrease in 11-KT was also due to disruption in testicular function (Sharpe et al., 2007).

In the present study, we investigated the E_2/11-KT ratio to indirectly evaluate aromatase activity, which is a key factor in testicular development (Mandich et al., 2007; Quignot et al., 2012). E_2 levels in goldfish exposed to VZ remained unchanged, while the E_2/11-KT ratio decreased and increased in goldfish exposed to 100 and 800 µg/L VZ following 30 days of exposure, respectively. The observed changes in the E_2/11-KT are likely to be due to 11-KT level rather than changes in aromatase activity, since *CYP19a1a* and *CYP19a1b* mRNA were not affected. This is consistent with the lack of change observed in *Vtg* and *ERα* mRNA in the liver of goldfish exposed to VZ as shown in previous studies on fathead minnows and rats (Martinović et al., 2008; Loutchanwoot et al., 2008). However some studies indicated up-regulation of cytochrome P450 genes following exposure of male fathead minnow to an anti-androgen (ketoconazole) which was associated with decrease in circulating androgen level in blood plasma (Villeneuve et al., 2007; Ankley et al., 2012). In contrast, a decrease in the E_2/T ratio in male rats exposed to VZ have shown to be related to both decreases and increases in E_2 and T levels associated with increases in testicular aromatase activity and *ERα* mRNA (Quignot et al., 2012). Therefore, there may be other contributing factors for VZ-related alternations in the E_2/11-KT level as there is evidence that CYP19a1b regulates estrogenic potency of E_2 to induce Vtg production in the liver through ER subtypes (Ishibashi et al., 2001; Kishida et al., 2001; Marlatt et al., 2008; Nelson and Habibi, 2010).

Fish AR localized in both brain (hypothalamus and pituitary) and testis shows high affinity for 11-KT (Olsson et al., 2005; Hossain et al., 2008) and may be involved in regulation of LH release by androgens (Kah, 2009). The observed decrease in *AR* mRNA in the brain of goldfish exposed to VZ may be due to 11-KT reduction, since it was shown previously that changes in *AR* mRNA correlate with the levels of sex steroids (Burmeister et al., 2007; Liu et al., 2009). At day 30, *AR* mRNA in the brain was increased in goldfish exposed to 100 µg/L VZ, which is consistent with the observed increase in plasma 11-KT level. Anti-androgenic activity of VZ has shown to be mediated by competitive binding to AR in mammals (Kelce et al., 1994; Wong et al., 1995; Andersen et al., 2002). In fish, VZ shows no competitive binding affinity to the brain

cytosolic extracts AR compared to T (Makynen et al., 2000). However, testicular *AR* mRNA increased in fathead minnows following exposure to VZ (Martinović et al., 2008, present study). In contrast, the present study showed decrease in the brain *AR* mRNA following 7 days of exposure to 100–800 μg/L, which was increased in goldfish exposed to 100 μg/L at day 30 of exposure. These results suggest that the brain AR might mediate VZ-related disruption in 11-KT biosynthesis. Since 11-KT is major androgen in fish (Olsson et al., 2005; Hossain et al., 2008; Kah, 2009), further studies should consider competitive binding affinity of VZ to AR compared to 11-KT (and may be T as precursor of 11-KT) following an acute and chronic exposure for better understanding of VZ mode of action to disrupt fish reproduction.

In conclusion, the present study provides novel information on the mechanism of VZ-related disruption of testicular function in goldfish. In agreement with our previous study, the findings provide evidence that VZ interferes with both neuroendocrine and endocrine functions to impair male fertility. The adverse effects of VZ depend on the concentration and duration of exposure (Fig. 8) and are mediated through effects on the hormones of the brain–pituitary–testicular axis. The outcome is changes spermatogenesis and testicular development. The present findings provide a framework for a better understanding of the endocrine disruptive effects of VZ in goldfish and other vertebrate species.

Conflict of interest

The authors have declared that no competing and conflict of interests exist.

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ALTERATIONS IN TRANSCRIPTS OF REPRODUCTIVE NEUROENDOCRINE HORMONES AND ANDROGEN RECEPTOR IN GOLDFISH EXPOSED TO VINCLOZOLIN, FLUTAMIDE, TESTOSTERONE AND THEIR COMBINATIONS

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ABSTRACT

Vinclozolin (VZ) is a pesticide that acts as an anti-androgen to impair reproduction in mammals. However, VZ effects on fish reproduction are largely unknown. We have established a combination exposure protocol using VZ (30 and 100 μg/L), anti-androgen flutamide (Flu, 300 μg/L) and androgen testosterone (T, 1 μg/L) to study adverse effects of VZ reproductive endocrine system in adult goldfish. Kisspeptin (kiss-1 and kiss-2) and its receptor (gpr54), salmon gonadotropin releasing hormone (gnrh3) and androgen receptor (ar) mRNA levels in the mid-brain and testicular luteinizing hormone receptor (lhr) were studied and compared with control following 10 d of exposure. kiss-1 mRNA level was increased in goldfish exposed to 100 μg/L VZ and Flu, while kiss-2 mRNA was increased following exposure to Flu and combinations of 30 μg/L VZ with Flu, 100 μg/L VZ with T, and Flu with T. gpr54 mRNA was increased in goldfish exposed to Flu and combination of 30 μg/L VZ with Flu and 100 μg/L VZ with T. gnrh3 level was increased in goldfish exposed to 100 μg/L VZ, Flu and combinations of 30 μg/L VZ with Flu, 100 μg/L VZ with T, and Flu with T. The brain ar mRNA level was increased in goldfish exposed to Flu and combinations of 30 μg/L VZ with Flu, 100 μg/L VZ with T, and Flu with T. Testicular lhr mRNA level was observed in goldfish exposed to Flu and combination of 30 μg/L VZ with Flu. These results suggest that VZ and Flu are capable of interfering with kiss-1/gpr54 and gnrh3 functions to control pituitary and testicular hormonal function in goldfish. Since previous studies show that the effects of VZ to stimulate 11-KT production in fish similar to Flu effects, this study suggests that the brain ar mediates VZ-related disruption in 11-KT biosynthesis in goldfish.

Keywords: *androgen receptor; flutamide; kisspeptin; luteinizing hormone receptor; salmon gonadotropin-releasing hormone; testosterone*

Introduction

In our environment, there are a large number of natural and synthetic compounds known or suspected to mimic or interfere with reproductive endocrine system (Bergman et al., 2012). There are sufficient evidences supporting the hypothesis that endocrine disrupting chemicals (EDCs) contributes to male reproductive disorders in fish (Jobling et al., 2002; Aravindakshan et al., 2004; Jeffries et al., 2008; Marchand et al., 2008; Jordan et al., 2012). Compared to estrogenic EDCs, there is a significant knowledge gaps underlying association between male infertility and exposure to EDCs that interfere with androgen signaling (Villeneuve et al., 2007; Martinovic et al., 2008; Hatef et al., 2012a,b; Golshan et al., 2014).

Vinclozolin (VZ) is a dicarboximide fungicide widely used for control of diseases in fruits and vegetables (Pothuluri et al., 2000). In both mammals and fish, VZ impairs male reproduction through stimulation of androgen production in the testis which leads to 17β-estradiol (E_2)/ testosterone (T) or 11-ketotestosterone (11-KT) imbalance (Gray et al., 1999; Kubota et al., 2003; Loutchanwoot et al., 2008; Martinovic et al., 2008; Eustache et al., 2009; Quignot et al., 2012; Hatef et al., 2012b; Golshan et al., 2014).

At spawning, pituitary luteinizing hormone (LH) controls testicular hormonal function to release mature spermatozoa in fish (see reviews by Kah 2009; Van der Kraak 2009). Gonadotropin-releasing hormone (GnRH) and the feedback effects of steroid hormones control LH release from pituitary. Thus, EDCs can interfere with reproduction by their direct effects on hypothalamus-pituitary-testis (HPT) hormonal functions or by their indirect effects through alternations in feedback effects of sex steroids. We have recently reported a significant increase in 11-KT level in VZ-treated goldfish which lead to decrease in sperm quality (Hatef et al., 2012; Golshan et al., 2014). Current data suggest that VZ affects hormonal functions of HPT to alter androgen production in fish (Villeneuve et al., 2007; Martinovic et al., 2008; Golshan et al., 2014) similar to those in mammals (Kubota et al., 2003; Loutchanwoot et al., 2008; Quignot et al., 2012). Our results also suggest that VZ alters the brain androgen receptor (*ar*) mRNA levels. However, in contrast to mammals (Kelce et al., 1994; Wong et al., 1995), VZ binding affinity to the brain AR has not been seen in fish (Sperry and Thomas 1999; Makynen et al., 2000). Overall, the effects of VZ to stimulate testicular androgen production are largely unknown in fish.

Kisspeptin-1 (Kiss-1) is a neuroendocrine peptide that regulates reproduction in vertebrates (see review by Pinilla et al., 2012). At least two isoforms of *kiss-1* (*kiss-1* and *kiss-2*) have been identified in fish (see reviews by Akazome et al., 2010; Gopurappilly et al., 2013) including goldfish (Li et al., 2009; Yang et al., 2010). It has shown that *kiss-1* controls GnRH and LH release from hypothalamus and pituitary, respectively, through activation of its receptor (*gpr54*) in goldfish (Li et al., 2009; Yang et al., 2010; Chang et al., 2012). In mice and rats, sex steroids affects on *Kiss-1* mRNA levels during adulthood and KISS-1 alters mRNA levels of sex steroid receptors (Smith et al., 2005; Adachi et al., 2007; Kauffman et al., 2007; Homma et al., 2009; Clarkson et al., 2012). It has been observed that mixture of estrogenic EDCs, estradiol benzoate or bisphenol A decrease or increase Kiss-1 and Gpr54 mRNA levels in rats, mice, ewes (Bellingham et al., 2009; Navarro et al., 2009; Panzica et al., 2009). These suggest that EDCs may disrupt reproductive endocrine system in mammals through KISS-1/GPR54 system. However, the effects of EDCs on Kiss-1/Gpr54 system have not been investigated in fish.

In the present study, we established a combination protocol to better understand the effects of VZ on reproductive endocrine system in goldfish (*Carassius auratus*). Adult male goldfish were exposed to VZ, flutamide (Flu), testosterone (T) and their combinations for 10 d and alternations in *kiss-1*, *kiss-2*, *gpr54*, *gnrh3*, and *ar* mRNA levels in the mid-brain and testicular *lhr* were studied. This study shows differences among VZ, Flu and T to alter reproductive endocrine system in goldfish. Considering anti-androgenic activity of Flu and androgenic activity of T, this study provides new information to better understand whether VZ acts as an anti-androgen to disrupt fish reproduction.

Materials and methods

Goldfish and treatments: experimental design and sampling

Adult male goldfish (mean body mass of 32 g and total length 13 cm) at stage before spermiation were exposed to vinclozolin (30 and 100 μg/L VZ), flutamide as anti-androgen (300 μg/L Flu) and testosterone as androgen (1 μg/L T) for a period of 10 d. Groups of

goldfish were also exposed to mixtures of these compounds including combination of 30 or 100 μg/L VZ with Flu, combination of 30 or 100 μg/L VZ with T, and combination of Flu with T. The aforementioned doses were selected based on previously published protocols (Iwamatsu et al., 2006; Filby et al., 2007; Hatef et al., 2012b; Golshan et al., 2014). All chemicals were dissolved in acetone, therefore one group of goldfish was exposed to acetone as solvent control. Flu was firstly dissolved in acetone under aeration at room temperature for about 1 h. After evaporation of the acetone, dechlorinated water was added to the glass vessel and the mixture was left to stir at room temperature for 24 h to ensure solubilisation of Flu before use. All experimental groups were kept under same conditions: the aquaria (each 40 L), temperatures (21 °C) and photoperiod (14 h light/10 h dark). The aquaria water was renewed every day. The fish were fed once a day with commercial food. Samples of mid-brain and testis were collected in an RNAse free tube, frozen immediately in liquid nitrogen and kept at -80˚C until use. Previous studies have shown expression of *gnrh3*, *ar*, *kiss-1*, *kiss-2* and *gpr54* in the mid-brain (see reviews by Kah, 2009; Akazome et al., 2010; Gopurappilly et al., 2013). There were no differences in fish weight and length among treatments ($p > 0.05$, data are not shown). This study was carried out in strict accordance with the recommendations in the guide for the care, protection and use of laboratory animals of the Canadian Council approved by the ethics committee of the university animal care committees. All surgery was performed under 2-phenoxyethanol anesthesia, and all efforts were made to minimize suffering.

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from each tissue sample 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, Nanodrop, USA) and RNA quality was verified by A260 nm/A280 nm ratios between 1.8 and 2 and A230 nm/A260 nm ratios >2. cDNA was synthesized from 4 μg of 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, Madison, WI, USA) following the manufacturer's instructions. Two μL oligo $(dT)_{18}$ primer (500 μg/mL) was added to each samples 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 DTT (100 mM), 0.4 μL dNTP (100 mM) (Cat. No. dNTP-01, UBI Life Science, Canada) and 0.7 μL M-MLV (200 U/μL) and nuclease free water 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 iQ cycler. The reaction was deactivated by heating at 70°C for 15 min. A negative control was run for each sample. Each 18 μL reaction was diluted 3-fold in nuclease free water and used for quantitative real time PCR assay. iCycler iQ Real-time PCR Detection System (Bio-Rad Lab. Inc.) was used for studying mRNA levels with the following condition per reaction: 1 μL of diluted cDNA, 0.26 μL of forward and reverse primers, 12.5 μL SYBR Green PCR Master Mix (Qiagen Mississauga, Canada) and ultrapure distilled water (Invitrogen) to a total volume of 25 μL. Table 1 shows the sequences of specific primers used in this study. PCR was run in the following cycling conditions: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C, appropriate annealing temperatures for 31 s. 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 non-specific products. A single melt curve was observed for each primer set in all quantitative PCR reactions. No amplification product was also observed in non-template controls as well as no primer-dimer formations. Each sample was run in triplicate and the mean threshold cycles (determined by the linear portion of the fluorescence absorbance curve) were used for the final calculation. The mRNA levels were

normalized to the mRNA level of β-actin or glyceraldehyde-3- phosphate dehydrogenase (GAPDH) using the standard $2^{-\Delta\Delta Ct}$ method.

Statistical analyses

Homogeneity of variance and normal distribution of data were tested using Levene 's test and Kolmogorov–Smirnov 's test, respectively. As needed, data were transformed to meet assumptions of normality and homoscedasticity. Duncan post *hoc* test was used in conjunction with an ANOVA to find which means are significantly different from one another at each sampling time (alpha = 0.05). All data are presented as mean ± standard error of mean (SEM).

Table 1. *Primer sequences and annealing temperature applied for studying alternations in mRNA transcript of selected genes using qRT-PCR in the present study.*

Genes	Forward Primer	Reverse Primer	Annealing temperature (˚C)
kiss-1	CAGATCCTCAGCGAAACACA	GCAAGCATGTTCTGCTCTCT	56
kiss-2	GCAGTTGATGTTGAGGGGAA	ATGCAACCGAGGCCATGAAA	56
gpr54	TTTGGGGACTTCATGTGTCG	ATCTGTGGTGTTCGATGACG	56
gnrh3	TGGTCCAGCTCCTAATGCTA	AACACTTCTCTTTCCACCCG	57
lhr	CCTCTGCATCGGTGTGTATC	TAGACAGATAGTTCGCCGCC	60
ar	GATGAAAGGTCCAGAGGAGG	ACTGTGAGTGGAACGTCAGG	57
gapdh	TGATGCTGGTGCCCTGTATGTAGT	TGTCCTGGTTGACTCCCATCACAA	57
β-actin	GTTTTGCTGGAGATGATGCC	TTCTGTCCCATGCCAACCAT	57

Kisspeptins (*kiss1* and *kiss2*), kisspeptin receptor (*gpr54*), Salmon gonadotropin-releasing hormone (*gnrh3*), Luteinizing hormone receptor (*lhr*), Androgen receptor (*ar*). Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) was used as house keeping gene for testicular *ar* and *lhr*. β-actin was used for mRNA analysis of genes in the brain (*kiss1*, *kiss2*, *gpr54*, *gnrh3* and *ar*).

Results

The mid-brain *gnrh3*, *kiss-1*, *kiss-2*, *gpr54*, and *ar* mRNA levels

Compared to control, *kiss-1* mRNA level was increased in goldfish exposed to 100 μg/L VZ and Flu ($P < 0.05$; Fig. 1A). However, *kiss-1* mRNA level remained unchanged in goldfish exposed to 30 μg/L VZ, T and combination of 30 or 100 μg/L VZ with T, combination of Flu with T, and combination of 30 μg/L VZ with Flu ($P > 0.05$). Compared to control, *kiss-2* mRNA level was increased in goldfish exposed to Flu, combination of 30 μg/L VZ with Flu, combination of 100 μg/L VZ with T, and combination of Flu with T ($P < 0.05$; Fig. 1B). *kiss-2* mRNA level remained unchanged in goldfish exposed to 30 or 100 μg/L VZ, T and combination of 30 μg/L VZ with T compared to control ($P > 0.05$). *gpr54* mRNA level was increased in goldfish exposed to Flu, combination of 30 μg/L VZ with Flu and combination of 100 μg/L VZ with T ($P < 0.05$; Fig. 1C). Compared to control, *gpr54* mRNA remained unchanged in goldfish exposed to 30 or 100 μg/L VZ, T, combination of 30 μg/L VZ with T, and combination of Flu with T ($P > 0.05$).

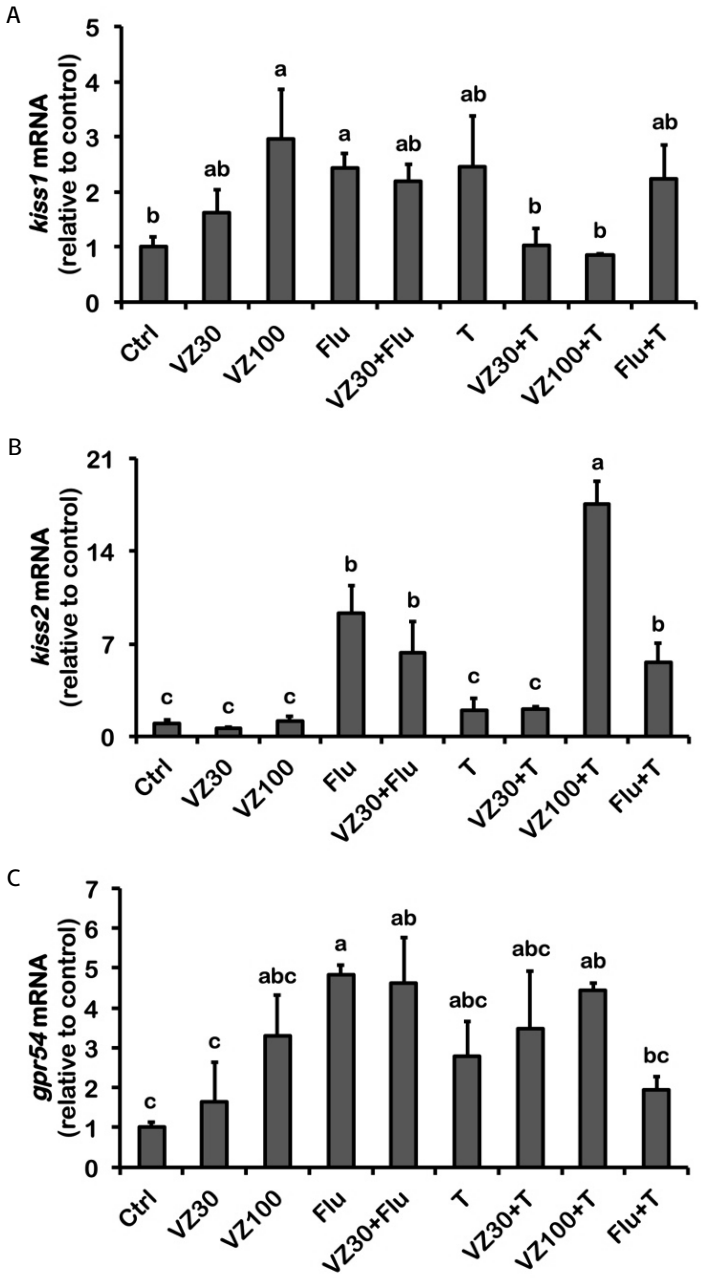

Fig. 1. *Alterations in kisspeptins (kiss-1, A; kiss-2, B) and their receptor (gpr54, C) mRNA levels in the mid-brain of goldfish (Carassius auratus) exposed to vinclozolin (VZ), anti-androgen flutamide (Flu), androgen testosterone (T) and their combinations for a period of 10 d. Adult male goldfish were exposed to 30 or 100 μg/L VZ (VZ30 and VZ100, respectively), 300 μg/L Flu (Flu), 1 μg/L T (T), combination of 30 μg/L VZ with 300 μg/L Flu (VZ30+Flu), combination of 30 or 100 μg/L VZ with 1 μg/L T (VZ30+T and VZ100+T), and combination of 300 μg/L Flu with 1 μg/L T (Flu+T). Data were normalized to the internal control and is expressed as mean ± SEM (n = 6). Values with different superscripts are significantly different (P < 0.05, ANOVA with Duncan post hoc test).*

gnrh3 mRNA level was increased in goldfish exposed to 100 μg/L VZ, Flu and combinations of 30 μg/L VZ with Flu, combination of 100 μg/L VZ with T, and combination of Flu with T compared to control ($P < 0.05$; Fig. 2). *gnrh3* mRNA level remained unchanged in goldfish exposed to 30 μg/L VZ, T and combination of 30 μg/L VZ with T, compared to control ($P > 0.05$).

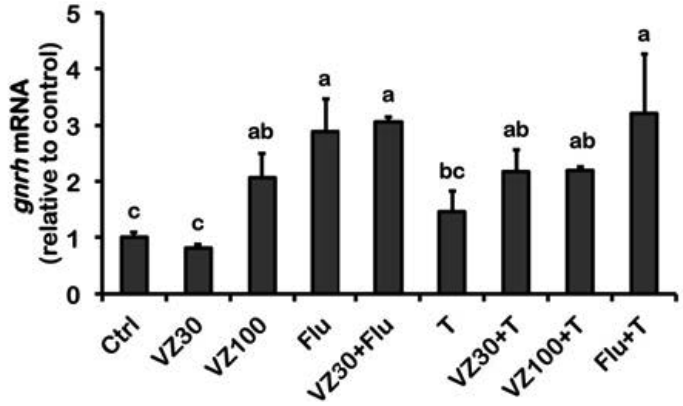

***Fig. 2**. Alterations in Salmon gonadotropin-releasing hormone (gnrh3) mRNA levels in the mid-brain of goldfish (Carassius auratus) exposed to vinclozolin (VZ), anti-androgen flutamide (Flu), androgen testosterone (T) and their combinations for a period of 10 d. Adult male goldfish were exposed to 30 or 100 μg/L VZ (VZ30 and VZ100, respectively), 300 μg/L Flu (Flu), 1 μg/L T (T), combination of 30 μg/L VZ with 300 μg/L Flu (VZ30+Flu), combination of 30 or 100 μg/L VZ with 1 μg/L T (VZ30+T and VZ100+T, respectively) and combination of 300 μg/L Flu with 1 μg/L T (Flu+T). Data were normalized to the internal control and is expressed as mean ± SEM (n = 6). Values with different superscripts are significantly different (P < 0.05, ANOVA with Duncan post hoc test).*

Compared to control, *ar* mRNA level was increased in goldfish exposed to Flu, combination of 30 μg/L VZ with Flu, combination of 100 μg/L VZ with T, and combination of Flu with T ($P < 0.05$; Fig. 3). However, ar mRNA level did not differ in goldfish exposed to 30 or 100 μg/L VZ, T and combination of 30 μg/L VZ with T ($P > 0.05$).

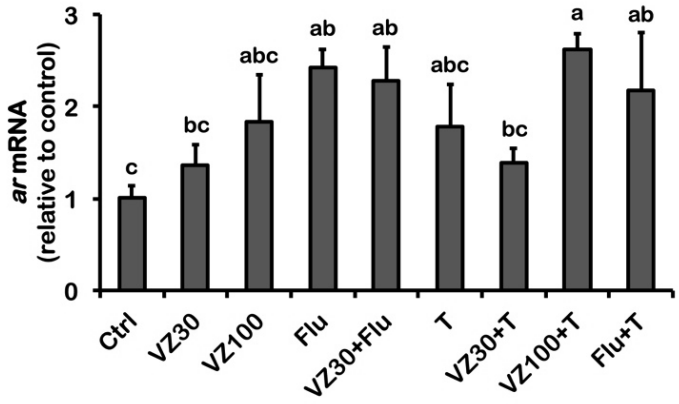

Fig. 3. *Alterations in the mid-brain androgen receptor (ar) mRNA levels in goldfish (Carassius auratus) exposed to vinclozolin (VZ), anti-androgen flutamide (Flu), androgen testosterone (T) and their combinations for a period of 10 d. Adult male goldfish were exposed to 30 or 100 μg/L VZ (VZ30 and VZ100, respectively), 300 μg/L Flu (Flu), 1 μg/L T (T), combination of 30 μg/L VZ with 300 μg/L Flu (VZ30+Flu), combination of 30 or 100 μg/L VZ with 1 μg/L T (VZ30+T and VZ100+T, respectively), and combination of 300 μg/L Flu with 1 μg/L T (Flu+T). Data were normalized to the internal control and is expressed as mean ± SEM (n = 6). Values with different superscripts are significantly different (P < 0.05, ANOVA with Duncan post hoc test).*

Testicular *Ihr* mRNA levels

Significant increase in testicular *Ihr* mRNA level was observed in goldfish exposed to Flu and combination of 30 μg/L VZ with Flu, compared to that of control ($P < 0.05$; Fig. 4). However, testicular *Ihr* mRNA level remained unchanged in goldfish exposed to 30 or 100 μg/L VZ, T, and combinations of VZ or Flu with T ($P > 0.05$).

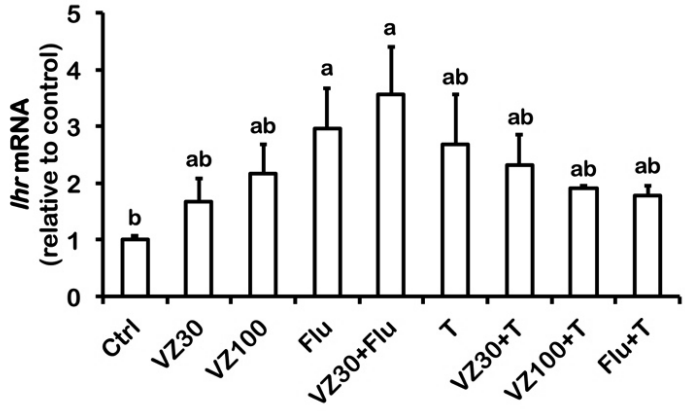

Fig. 4. *Alterations in testicular luteinizing hormone receptor (lhr) mRNA levels goldfish (Carassius auratus) exposed to vinclozolin (VZ), anti-androgen flutamide (Flu), androgen testosterone (T) and their combinations for a period of 10 d. Adult male goldfish were exposed to 30 or 100 μg/L VZ (VZ30 and VZ100, respectively), 300 μg/L Flu (Flu), 1 μg/L T (T), combination of 30 μg/L VZ with 300 μg/L Flu (VZ30+Flu), combination of 30 or 100 μg/L VZ with 1 μg/L T (VZ30+T and VZ100+T, respectively) and combination of 300 μg/L Flu with 1 μg/L T (Flu+T). Data were normalized to the internal control and is expressed as mean ± SEM (n = 6). Values with different superscripts are significantly different (P < 0.05, ANOVA with Duncan post hoc test).*

Discussion

We have recently observed that VZ stimulates 11-KT production associated with an increase in circulating LH level which results in diminished sperm quality in male goldfish (Hatef et al., 2012, Golshan et al., 2014). The present study provides new information about the potential effects of VZ on upstream neuroendocrine regulators of pituitary and testicular hormonal functions. To better understand the effects of VZ on reproductive endocrine system, we have established a combination protocol in which goldfish were exposed to VZ, Flu, T and their combinations. In this study, androgen T and anti-androgen Flu were applied at concentrations that do not exhibit *aromatizable* activity (Hagino et al., 2001; Iwamatsu et al., 2006).

The brain *ar* mRNA level increased in goldfish exposed to Flu. This is due to the facts that Flu stimulates androgen production in fish (Rajakumar et al., 2012) similar to mammals (O'Connor et al., 2002; Ohsako et al., 2003; Vo et al., 2009; Place et al., 2011) and *ar* gene expression positively correlates with circulating androgen levels (Burmeister et al., 2007). It is worth to note that both T and 11-KT show high affinity to bind to AR in fish (Sperry and Thomas 1999; Todo et al., 1999; Hossain et al., 2008). In contrast to T exposed goldfish, the brain *ar* mRNA level increased in goldfish exposed to combination of 100 μg/L VZ or Flu with T compared to control. These interesting results suggest that VZ, similar to Flu, interferes with negative feedback of T through the brain *ar*. However the brain *ar* mRNA level did not change in goldfish exposed to 30 or 100 μg/L VZ. This might be due to time-dependent effects of VZ to stimulate androgen production in goldfish (Golshan et al., 2014). Increased *ar* mRNA level in the brain of goldfish exposed to combination of 30 μg/L VZ with Flu suggest that both VZ and Flu acts in similar way. However, VZ competitive binding affinity to the brain AR has been only observed in mammals (Wong et al., 1995). In fish, studies have shown binding affinity of metabolites of VZ (M1 and M2) to AR (Sperry and Thomas 1999; Makynen et al., 2000). We also observed that testicular *ar* mRNA remained unchanged following exposure of goldfish to

VZ, Flu, T and their combinations (data are not shown), which consistent with our previous study (Golshan et al., 2014). This study suggests that the brain AR is major target for the effects of VZ and Flu on HPT hormonal functions.

gnrh3 mRNA level increased in the brain of goldfish exposed to 100 μg/L VZ and Flu which consistent with previous studies in fish (Rajakumar et al., 2012; Golshan et al., 2014) and rats (Ohsako et al., 2003). Similarly, *gnrh3* mRNA level increased in goldfish exposed to combination of 30 μg/L VZ with Flu. These data suggest that VZ, similar to Flu, acts on hypothalamus to stimulate LH production and release from pituitary leading to induce androgen production. It is worth to note that *gnrh3* mRNA level and circulating LH and 11-KT levels positively correlate in fish (Khakoo et al., 1994; Klausen et al., 2002; Golshan et al., 2014). *gnrh3* mRNA level remained unchanged in T treated goldfish, but it was increased in goldfish exposed to combination of either 100 μg/L VZ or Flu with T. These results suggest that VZ and Flu are capable of interfering with T feedback mechanism at the level of hypothalamus (see review by Habibi and Huggard 1998).

In this study, the brain *kiss-1*, *kiss-2* and *gpr54* mRNA levels increased in Flu treated goldfish. However, VZ increased *kiss-1* mRNA level without effects on *kiss-2* and *gpr54* mRNA levels. Increased *kiss-1*, *kiss-2* or *gpr54* mRNA levels were associated with increase in *gnrh3* mRNA level and consistent with circulating LH level in Flu or VZ treated goldfish (Rajakumar et al., 2012; Golshan et al., 2014). Considering increases in *kiss-2* and *gpr54* mRNA levels in goldfish exposed to combination of 30 μg/L VZ with Flu, these results suggest that VZ, similar to Flu, acts on Kiss-1/Gpr54 system to interfere with HPT hormonal functions. It is possible because Kiss-1 and AR are co-localized in the mid-brain of goldfish (Gelinas and Callard, 1997; Li et al., 2009; Yang et al., 2010). However, we observed that changes in *kiss-1*, *kiss-2*, *gpr54* mRNA levels differ between Flu and VZ treated goldfish suggesting that Flu and VZ have different effects via ligand or receptor. In contrast to Flu and VZ, *kiss-1*, *kiss-2* and *gpr54* mRNA levels remained unchanged in T treated goldfish associated with no change in *gnrh3* and *lhr* mRNA level. However combination of Flu with T and 100 μg/L VZ with T increased *kiss-2* or *gpr54* mRNA levels. These suggest that negative feedback of T on hypothalamus and pituitary hormonal functions is not mediated through *kiss-1,2/gpr54* system in goldfish.

In this study, testicular *lhr* mRNA level increased in Flu treated goldfish, but it was unchanged in goldfish exposed to 30 or 100 μg/L VZ. However, previous studies have shown increase in testicular *lhr* mRNA level in fathead minnow exposed to 100 μg/L VZ (Villeneuve et al., 2007) and in goldfish exposed to 400 μg/L VZ following 21 and 7 d of exposure (Golshan et al., 2014). This might be due to the dose- and time-dependent effect of VZ to stimulate LH release from pituitary and thus affect testicular *lhr* mRNA level.

In conclusion, the mid-brain *kiss-1* and *gnrh3* mRNA levels were increased in goldfish exposed to 100 μg/L VZ. In Flu treated goldfish, the mid-brain *kiss-1*, *kiss-2*, *gpr54*, *gnrh3*, and *ar* mRNA levels were increased associated with an increase in testicular *lhr* mRNA level. In contrast, all of these parameters remained unchanged in T treated goldfish. These results suggest that VZ and Flu are capable of interfering with *kiss-1* and *gnrh3* functions to control pituitary and testicular hormonal function in goldfish. Combination exposure resulted in increase in the brain *kiss-2*, *gnrh3* and *ar* when either 100 μg/L VZ or Flu was combined with T. The mid-brain *gpr54* mRNA level was increased in the brain of goldfish exposed to combination of VZ with T. These suggest that VZ, very similar to Flu, acts as anti-androgen to impair fish reproduction. Results of the present study and those of Hatef et al. (2012) and Golshan et al. (2014) suggest that VZ acts on *kiss-1* to stimulate *gnrh3* in the hypothalamus which in turn increases LH production and release from pituitary. These changes result in induction of 11-KT production associated with an increase in mRNA of genes encoding enzymes in steroidogenesis. The brain *ar* mediates VZ-related disruption in 11-KT biosynthesis in goldfish.

Conflict of interest

The authors have declared that no competing and conflict of interests exist.

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CHAPTER 3

REPRODUCTIVE ENDOCRINE SYSTEM IN GOLDFISH EXPOSED TO DI-(2-ETHYLHEXYL)-PHTHALATE, *IN VIVO*

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Di-(2-ethylhexyl)-phthalate disrupts pituitary and testicular hormonal functions to reduce sperm quality in mature goldfish

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Vitellogenin

ABSTRACT

Di-(2-ethylhexyl) phthalate (DEHP) interferes with male reproductive endocrine system in mammals, however its effects on fish reproduction are largely unknown. We evaluated sperm quality and investigated reproductive endocrine system in mature goldfish (*Carassius auratus*) exposed to nominal 1, 10, and 100 μg/L DEHP. To examine DEHP estrogenic activity, one group of goldfish was exposed to 17β-estradiol (5 μg/L E_2) for comparison. Following 30 d of exposure, sperm production was decreased and suppressed in DEHP and E_2 treated goldfish, respectively. Sperm motility and velocity were decreased in goldfish exposed to 100 and 10 μg/L DEHP at 15 s post-sperm activation, respectively. Compared to control, 11-ketotestosterone (11-KT) levels were decreased at 10 and 1 μg/L DEHP at day 15 and 30, respectively. In E_2 treated goldfish, 11-KT levels were decreased compared to control during the period of exposure. E_2 levels were increased in goldfish exposed to E_2, but remained unchanged in DEHP treated goldfish during the period of exposure. *StAR* mRNA levels encoding regulator of cholesterol transfer to steroidogenesis were decreased in DEHP and E_2 treated goldfish following 15 and 30 d of exposure, respectively. Luteinizing hormone (LH) levels were decreased in DEHP and E_2 treated goldfish following 15 and 30 d of exposure, respectively. In DEHP treated goldfish, *gnrh3*, *kiss1* and its receptor (*gpr54*) mRNA levels did not change during the experimental period. In E_2 treated goldfish, *gnrh3* mRNA levels were decreased at day 7, but *kiss1* and *gpr54* mRNA levels were increased at day 30 of exposure. The mRNA levels of genes encoding testicular LH and androgen receptors remained unchanged in DEHP and E_2 treated goldfish. In contrast to E_2 treated goldfish, vitellogenin production was not induced in DEHP treated goldfish and mRNA levels of genes with products mediating estrogenic effects remained unchanged or decreased. In conclusion, DEHP interferes with testis and pituitary hormonal functions to reduce sperm quality in goldfish and does not exhibit estrogenic activity.

1. Introduction

Di-(2-ethylhexyl) phthalate (DEHP) is a plasticizer not chemically bound to polymer matrices and can therefore leak out into the surrounding environment. It is used as a non-PVC material in the plastic industries and has been detected in the construction sectors, packaging products, medical devices, pharmaceuticals, cosmetics and personal care products (European Communities, 2008; Kastner et al., 2012; Magdouli et al., 2013). The U.S. Environmental Protection Agency and the World Health Organization have established a DEHP safety concentration limit in drinking water to be 6 and 8 μg/L, respectively (World Health Organization, 1996; Clark et al., 2003). DEHP is measured below 1 and 10 μg/L in drinking

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water and surface water, respectively, and up to 98–219 μg/L in polluted area (Fromme et al., 2002; Yuwatini et al., 2006; Khan and Jung, 2008).

It has been observed that DEHP decreases fertilization rate when male zebrafish are injected with 5000 mg/kg b.w. DEHP for 10 d (Uren-Webster et al., 2010) or exposed to 0.2–20 μg/L DEHP for 21 d (Corradetti et al., 2013). Male fertility also decreases in marine medaka exposed to 100 and 500 μg/L DEHP from hatching to maturity stage (Ye et al., 2014). However, it is still unknown whether DEHP-reduced male fertility is corresponding to DEHP effects on sperm production, motility and velocity, which are key determinants for male fertility in fish (Billard et al., 1995; Alavi and Cosson, 2006; Linhart et al., 2008). In mammals, DEHP-reduced male fertility is associated with decrease in sperm production (Andrade et al., 2006a; Foster, 2006; Lee et al., 2009).

Thibaut and Porte (2004) reported that DEHP inhibits 5α-dihydrotestosterone synthesis in fish *in vitro*; a potent androgen that binds to androgen receptor (*ar*). However, *in vivo* studies show no change in testosterone (T) levels in mature fathead minnow (Crago and Klaper, 2012) and zebrafish (Uren-Webster et al., 2010) exposed to DEHP associated with no changes in *StAR* mRNA levels that encodes the steroidogenic acute regulatory protein to control the transfer of cholesterol into the steroidogenesis (Stocco, 2001). Ye et al. (2014) also observed no changes in T levels in marine medaka exposed to DEHP, but *StAR* mRNA levels were increased. In contrast, Wang et al. (2013) observed the increase in T levels in rare minnow exposed to DEHP. Overall, inhibitory effects of DEHP on androgen production are largely unknown in fish and none of the former studies have investigated 11-ketotestosterone (11-KT) levels, which is the major androgen in spermatogenesis (Miura and Miura, 2003). In mammals, in prepubertal and *in utero* DEHP exposure decreases T levels at 10–750 mg/kg/d in male rats which was associated with changes in circulatory luteinizing hormone (LH) levels and *StAR* mRNA levels (Parks et al., 2000; Akingbemi et al., 2001, 2004; Ge et al., 2007; Howdeshell et al., 2007; Culty et al., 2008; Lee et al., 2009; Hannas et al., 2012).

Similar to mammals (Akingbemi et al., 2004; Andrade et al., 2006b), it has shown that DEHP also stimulates vitellogenin (*Vtg*) production or *vtg* mRNA in zebrafish (Uren-Webster et al., 2010), rare minnow (Wang et al., 2013) and marine medaka (Ye et al., 2014), associated with increases in 17β-estradiol (E_2) and mRNA levels of genes encoding estrogen receptor subtypes (*er*). In contrast, Crago and Klaper (2012) reported decreases in E_2 levels and no change in mRNA levels of *er* subtypes and liver *vtg* mRNA in fathead minnow exposed to DEHP. Thus, DEHP estrogenic activity is still unclear and may differ among fish species.

In this study, mature male goldfish (*Carassius auratus*) were exposed to 1, 10, and 100 μg/L DEHP for a period of 30 d to study its effects on male fertility endpoints, sex steroid levels and neuroendocrine regulation of testicular hormonal functions. To study the effects of DEHP on male fertility endpoints, sperm production, motility and velocity were assessed following 30 d of exposure. 11-KT and E_2 levels were measured through the period of exposure to study whether DEHP alters testicular hormonal functions. *StAR* mRNA levels were analysed to understand whether changes in sex steroid levels correspond to transfer of cholesterol into the steroidogenesis (provided that a change in mRNA reflects a change in protein level). To understand DEHP effects on neuroendocrine regulation of testicular hormonal functions (Trudeau, 1997; Van der Kraak, 2009; Gopurappilly et al., 2013), we analysed circulatory LH levels and mRNA levels of genes encoding its receptor (*lhr*), salmon gonadotropin releasing hormone (*gnrh3*), and kisspeptin (*kiss1*) and its receptor (*gpr54*). Finally, estrogenic activity of DEHP was compared with E_2 treated goldfish by analyses of *vtg* expression and the liver *vtg* mRNA levels as well as the mRNA levels of genes encoding brain P450 aromatase (*cyp19a1b*) and liver *erα*.

2. Material and methods

2.1. Fish exposure and sampling

Mature male goldfish (2–3 year-old) were checked for their health and acclimatized for 14 d before exposure. Goldfish are synchronous spawners that produce groups of gametes several times throughout the spawning season. Final gamete maturation in goldfish highly depends on temperature. However, they could not spawn in captivity as submerged aquatic plants covering the bottom are required (Landau, 1992). Groups of goldfish were kept in 80 L aquaria without submerged aquatic plants under 12 h light/12 h dark photoperiod. Water temperature was 18 °C and 22 °C in 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 goldfish were fed once a day (3% body weight, b.w.) with commercial food (ZZN Vodnany, Czech Republic). This study was carried out in strict accordance with the recommendations in the Guide for the Care, Protection and Use of Laboratory Animals of the Ministry of Health of the Czech republic. The protocol was approved by the Central Ethics Committee of the Ministry of Agriculture of the Czech Republic (§17, article 1 of the low 246/1992 Sb). All surgery was performed using 2-phenoxyethanol (0.3 mL/L) anesthesia to minimize suffering.

The goldfish were exposed to DEHP at nominal concentrations of 1, 10 and 100 μg/L. One group of goldfish was exposed to the acetone (solvent control) and one group was kept in water (control). To account for exposure to the solvent, final acetone concentrations were constant among all treatment groups. The DEHP estrogenic activity was compared with a group of goldfish exposed to E_2 (nominal concentration 5 μg/L). Ten aquaria were used (2 per each group) and 3 individuals were sampled from each aquarium ($n = 6$ per each group). None of the examined parameters differed between the solvent control and control (data not shown). Thus, we have compared results of DEHP treated groups with those of the solvent control. The total length and body mass of goldfish were similar among the control, DEHP treated and E_2 treated groups (Table S1, Supporting information). Because DEHP degenerates over time (Staples et al., 1997), 80% of the water in each aquarium was renewed every 48 h and the solution was changed once per week. After recording body mass (±0.1 g) and total length (±1 mm), sperm, blood and tissue samples were collected according to Hatef et al. (2012). Each goldfish was sacrificed, carefully dissected and collected samples of brain, testis and liver were frozen in a liquid nitrogen. The gonadosomatic index (GSI = gonad weight/b.w. × 100) and hepatosomatic index (HSI = liver weight/b.w. × 100) were recorded. Blood samples were centrifuged (5000 rpm for 10 min at 4 °C) and collected plasma was stored together with tissues samples at −80 °C.

2.2. Sperm collection and quality

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. Sperm mass was measured per individual using a balance (±0.0001 g) and its value per kg b.w. was calculated. To evaluate sperm motility and velocity, sperm was directly activated at ratio 1:1000–2000 in a saline activation solution (NaCl 50 mM, KCl 5 mM, Tris 20 mM, pH 8.5) containing bovine serum albumin (0.1% w/v) to prevent sperm from sticking onto the glass 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. Sperm motility and velocity were analyzed based on the successive positions of sperm heads at various time points post-sperm activation (15, 30, 45 and 60 s) (for details see Hatef et al., 2010).

A DVD-recorder (SONY DVO-1000 MD, Japan) and a micro image analyzer (Olympus Micro Image 4.0.1. for Windows) were used to capture video frames for evaluating sperm motility (%) and curvilinear velocity (μm/s). Sperm velocity shows data of only motile sperm (~50–60 sperm per each record). For each individual, sperm motility was recorded two times and mean of the values were used in statistical analyses.

2.3. Determination of sex steroids

Blood plasma was diluted in a buffer provided by the Cayman EIA kit (Cayman, Michigan, USA) or by the Diasource EIA kit (Diasource, Nivelles, Belgium) for 11-KT and E_2 assays, respectively. Fifty micro liter of each control, 11-KT or E_2 standard and blood plasma were dispensed in separate wells. Antibody against 11-KT or E_2 and tracer (each 50 μL) were added into each well and incubated for 2 h at room temperature (RT). Following washing, adding the substrate solution (200 μL) and incubation of plates for 30–120 min at RT, the reaction was stopped by adding 100 μL of H_2SO_4. The optical density was read for 11-KT and E_2 at 405 and 450 nm, respectively. For E_2, sensitivity was 5 pg/mL, and intra-assay and inter-assay coefficients of variation were 4% and 6%, respectively, for a plasma concentration in the range of 100–250 pg/mL. For 11-KT, 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 values were used in statistical analyses.

2.4. Luteinizing hormone (LH) assay

Circulating LH levels were assessed by an ELISA method, previously established for LH assay in common carp (Kah et al., 1989). To validate the assay, serial dilutions of blood plasma were made together with a common carp standard. All were found to be parallel with the sensitivity of the assay in the range of 0.6–100 ng/mL and the intra- and inter-assay coefficients of variance at 5% and 9%, respectively.

2.5. Vitellogenin determination

Determination of *Vtg* induction was assessed using the Western blotting techniques. The blood plasma proteins were separated with SDS-PAGE and transferred to PVDF membrane according to Carnevali et al. (2010). The membrane was then incubated in blocking solution with rabbit anti-sea bream vitellogenin polyclonal antibody (Biosense Laboratories AS, Bergen, Norway) and the antibody reaction was visualized with chemiluminescent reagent.

2.6. Extraction of RNA, cDNA synthesis and quantitative 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 chloroform (200 μL) was added. After mixing, samples were centrifuged (12,000 × *g* for 15 min) and supernatants were transferred into new tubes containing an equal volume of isopropanol. The 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 concentration was estimated using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and its purity was verified by A260 nm/A280 nm and A230 nm/A260 nm ratios. An iScript cDNA Synthesis Kit was used to synthesize cDNA (Bio-Rad Lab). One μg RNA was mixed with 5× iScript reaction mix (4 μL), iScript reverse transcriptase (1 μL) and nuclease-free water (14 μL). The reaction mixture was incubated at 25 °C for 5 min and at 42 °C for 30 min and then deactivated by heating at 85 °C for 5 min. A negative control was run for each sample. The cDNA was used for qRT-PCR using an iQ5 iCycler thermal cycler (Bio-Rad Lab Inc., Milano, Italy) with SYBR Green. The reactions (10-fold diluted cDNA 1 μL, 2× iQ SYBR Green supermix containing SYBR Green as a fluorescent intercalating agent 5 μL, forward and reverse primers each 0.3 μL and RNase-free distilled water 3.4 μL) were set on a 96-well plate. Table 1 shows the primer sequences used for each gene. Primer efficiencies on serial dilutions of cDNA were all between 90% and 100%. 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. For each sample, qRT-PCR was run in triplicate to ensure consistency and the mean threshold cycles were used for the final calculation. No amplification product was observed in non-template controls and no primer-dimer formations were observed in the control templates. Relative expression levels were determined by normalizing to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or *β-actin* as the housekeeping gene (Livak and Schmittgen, 2001).

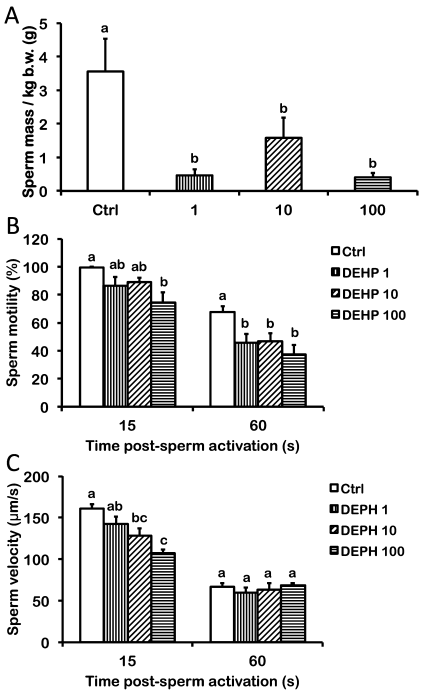

Fig. 1. Sperm production and motility kinetics in male goldfish exposed to di-(2-ethylhexyl)-phthalate (DEHP), *in vivo*. Mature male goldfish were exposed to nominal 1, 10 or 100 μg/L DEHP or 17β-estradiol (5 μg/L E_2) and sperm samples were collected following 30 d of exposure. No sperm was collected from E_2 treated goldfish, thus data were not shown. Sperm production was expressed as sperm mass per kg b.w. (A). Sperm motility (B) and velocity (C) were measured after activation in NaCl 50 mM, KCl 5 mM, Tris 20 mM, pH 8.5. Data are expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's *post hoc* test).

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

Genes	Forward primer	Reverse primer	Annealing temperature (°C)
lhr	CCTCTGCATCGGTGTGTATC	TAGACAGATAGTTCGCCGCC	60
ar	GATGAAAGGTCCAGAGGAGG	ACTGTGAGTGGAACGTCAGG	55
StAR	ATGGCTGGCAAACTGAGATCGAGA	TCCATGTTATCCACCAGCTCCTCA	57
cyp19a1a	TTGTGCGGGTTTGGATCAATGGTG	TTCCGATACACTGCAGACCCAGTT	55
cyp19a1b	AGGCGAGCGGGATGTAGAGT	CGTCCGATGTTCAGGATGAGG	58
vtg	GAAGTGCGCATGGTGGCTTGTATT	AGCTGCCATATCAGGAGCAGTGAT	55
erα	GAGGAAGAGTAGCAGCACTG	GGCTGTGTTTCTGTCGTGAG	55
gnrh3	TGGTCCAGCTCCTAATGCTA	AACACTTCTCTTTCCACCCG	57
kiss1	CAGATCCTCAGCGAAACACA	GCAAGCATGTTCTGCTCTCT	56
gpr54	TTTGGGGACTTCATGTGTCG	ATCTGTGGTGTTCGATGACG	56
gapdh	TGATGCTGGTGCCCTGTATGTAGT	TGTCCTGGTTGACTCCCATCACAA	57
β-actin	GTTTTGCTGGAGATGATGCC	TTCTGTCCCATGCCAACCAT	58

2.7. Statistical analysis

Data were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene's test). As needed, data were $\log_{10}$-transformed to meet assumptions of normality, and homoscedasticity. For understanding DEHP effects following acute and chronic exposure, we performed one-way ANOVA on each parameter followed by a Tukey's *post hoc* test at each sampling time separately. Alpha was set at 0.05. All data are presented as mean ± standard error of mean (S.E.M.).

3. Results

3.1. Effects of DEHP on GSI and HSI

No mortality was observed in any treatment during the experimental period. GSI in DEHP treated goldfish was similar to that of control, however it was decreased in E_2 treated goldfish following 15 and 30 d of exposure ($p < 0.05$; Table S1). There was no difference in HSI among the control, DEHP treated and E_2 treated goldfish ($p > 0.05$; Table S1).

3.2. Effects of DEHP on sperm production, motility and velocity

We could not collect sperm from one individual exposed to 1 and 10 μg/L DEHP, two individuals exposed to 100 μg/L DEHP, and all six individuals exposed to E_2. Sperm mass and its value per kg b.w. were decreased in goldfish exposed to 1–100 μg/L DEHP ($p < 0.05$; Figs. 1A and S1). Sperm motility was decreased in goldfish exposed to 100 μg/L DEHP evaluated at 15, 30 and 45 s post-sperm activation ($p < 0.05$, Figs. 1B and S1). At 60 s post-sperm activation, sperm motility was decreased in goldfish exposed to 1–100 μg/L DEHP ($p < 0.05$; Fig. 1B). Sperm velocity was decreased at 10 and 100 μg/L DEHP evaluated at 15 and 30 s post-sperm activation, respectively ($p < 0.05$), and remained unchanged evaluated at 45 and 60 s post-sperm activation ($p > 0.05$; Figs. 1C and S1).

3.3. Effects of DEHP on 11-KT and E_2 levels

The effects of DEHP on steroidogenesis were evaluated by analysis of 11-KT and E_2 levels (Fig. 2). Following 7 d of exposure, there were no differences in 11-KT levels between DEHP treated goldfish and the control ($p > 0.05$). However, 11-KT levels were lower in E_2 treated goldfish compared to those of control and 100 μg/L DEHP ($p < 0.05$). At day 15, 11-KT levels were decreased in goldfish exposed to 10 and 100 μg/L compared to the control ($p < 0.05$). At the same time, 11-KT levels in E_2 treated goldfish were lower than those of the control and DEHP treated goldfish ($p < 0.05$). Compared to the control, 11-KT levels were decreased in DEHP treated goldfish with the lowest levels observed at 1 μg/L DEHP following 30 d of exposure ($p < 0.05$). In goldfish exposed to E_2, 11-KT levels were lower than that of the control and 10 μg/L DEHP following

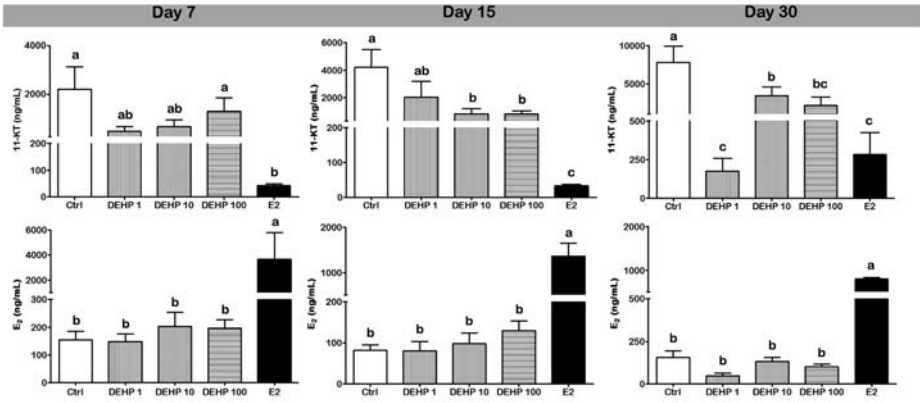

Fig. 2. Sex steroid levels in male goldfish exposed to di-(2-ethylhexyl)-phthalate (DEHP) and 17β-estradiol (E_2), *in vivo*. 11-ketotestosterone (11-KT) and E_2 were analyzed in the blood plasma samples of mature male goldfish exposed to nominal 1, 10 or 100 μg/L DEHP and to 5 μg/L E_2 following 7, 15 and 30 d of exposure. Data are expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's *post hoc* test).

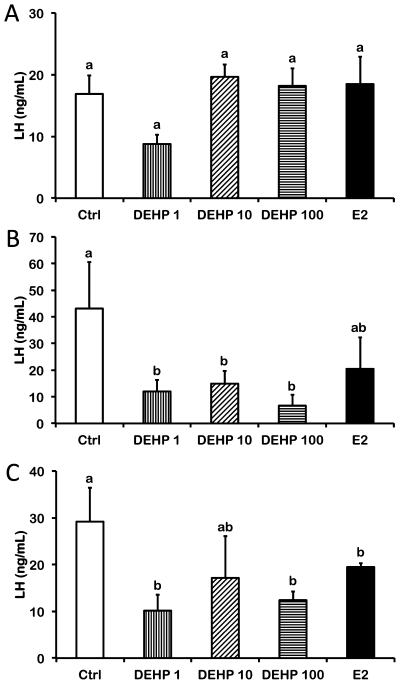

Fig. 3. Luteinizing hormone (LH) levels in male goldfish exposed to di-(2-ethylhexyl)-phthalate (DEHP) and 17β-estradiol (E_2), *in vivo*. Circulatory LH levels were measured in mature male goldfish exposed to nominal 1, 10 or 100 μg/L DEHP and to 5 μg/L E_2 following 7 (A), 15 (B) and 30 (C) d of exposure. Data are expressed as mean ± SEM ($n = 6$) and values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's *post hoc* test).

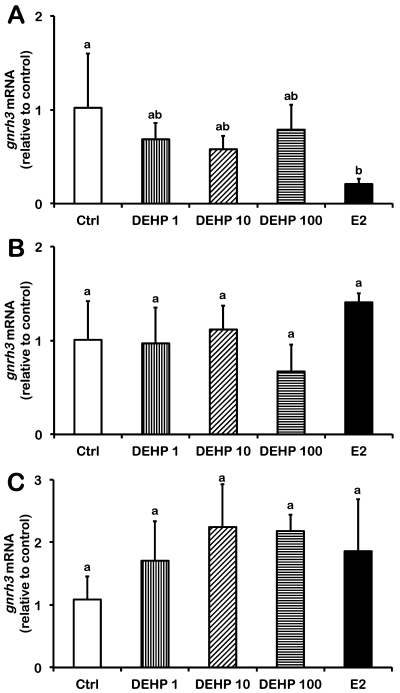

Fig. 4. Brain salmon gonadotropin-releasing hormone (*gnrh3*) mRNA levels in male goldfish exposed to di-(2-ethylhexyl)-phthalate (DEHP) and 17β-estradiol (E_2), *in vivo*. The brain samples were collected from mature male goldfish exposed to nominal 1, 10 or 100 μg/L DEHP and to 5 μg/L E_2 following 7 (A), 15 (B) and 30 (C) d of exposure to study alternations in *gnrh3* mRNA levels using a quantitative real-time PCR. Data were normalized to the internal control and are expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's *post hoc* test).

30 d of exposure ($p < 0.05$). There were no difference in E_2 levels between control and DEHP treated goldfish during the period of exposure ($p > 0.05$). As expected in E_2 treated goldfish, E_2 levels were increased compared to the control and DEHP treated goldfish at all time points during exposure ($p < 0.05$). There were no differences in E_2/11-KT ratio between the control and DEHP treated goldfish ($p > 0.05$; Fig. S2). However, it was higher in E_2 treated goldfish compared to those of the control and DEHP treated goldfish during the period of exposure ($p < 0.05$; Fig. S2).

3.4. Effects of DEHP on circulatory LH levels

At day 7 of exposure, circulatory LH levels remained unchanged in DEHP treated goldfish ($p > 0.05$; Fig. 3A). Circulatory LH levels were decreased in DEHP treated goldfish compared to the control following 15 d of exposure ($p < 0.05$; Fig. 3B). In E_2 treated goldfish, circulatory LH levels were not different from those of the control and DEHP treated goldfish following 7 and 15 d of exposure ($p > 0.05$; Fig. 3A and B). At day 30, circulatory LH levels were decreased in goldfish exposed to 1 and 100 μg/L DEHP and to E_2 compared to control ($p < 0.05$; Fig. 3C).

3.5. Effects of DEHP on the brain gnrh3, kiss1 and gpr54 mRNA levels

The mRNA levels of *gnrh3*, *kiss1* and *gpr54* did not change in DEHP treated goldfish during the period of exposure ($p > 0.05$; Figs. 4 and 5). In E_2 treated goldfish, *gnrh3* mRNA levels were decreased at day 7 ($p < 0.05$; Fig. 4), but *kiss1* and *gpr54* mRNA levels increased at day 30 of exposure compared to the control ($p < 0.05$; Fig. 5).

3.6. Effects of DEHP on mRNA of genes encoding androgen and LH receptors

Testicular *lhr* and *ar* mRNA levels showed no differences among the control and DEHP and E_2 treated goldfish during the period of exposure ($p > 0.05$; Fig. 6). The brain *ar* mRNA levels also remained unchanged in DEHP treated goldfish during the period of exposure ($p > 0.05$; Fig. 6). In E_2 treated goldfish, the brain *ar* mRNA levels were decreased following 7 d of exposure ($p < 0.05$), while it remained unchanged following 15 and 30 d of exposure ($p > 0.05$; Fig. 6).

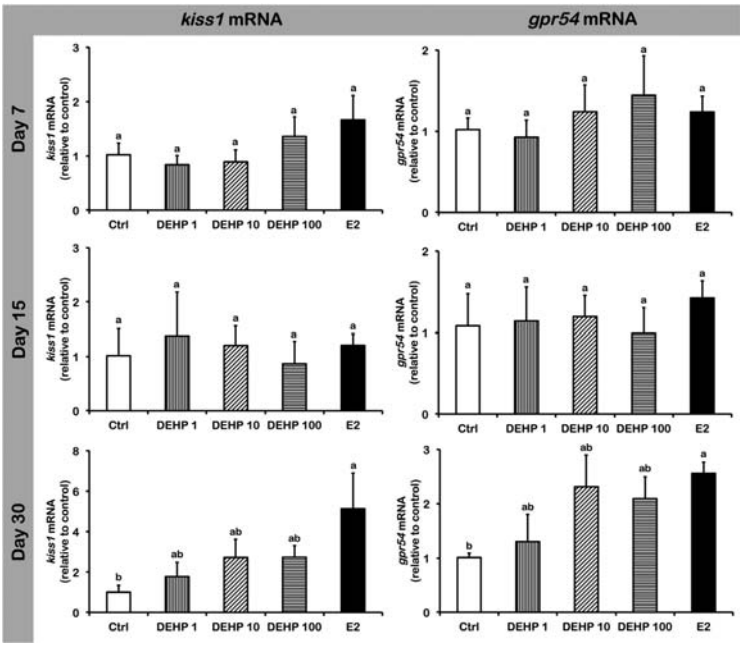

Fig. 5. mRNA levels of genes encoding brain kisspeptin (*kiss1*) and its receptor (*gpr54*) in male goldfish exposed to di-(2-ethylhexyl)-phthalate (DEHP) and 17β-estradiol (E_2), *in vivo*. The brain samples were collected from mature male goldfish exposed to nominal 1, 10 or 100 μg/L DEHP and to 5 μg/L E_2 following 7, 15 and 30 d of exposure to study alternations in *kiss1* and *gpr54* mRNA levels using a quantitative real-time PCR. Data were normalized to the internal control and are expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's *post hoc* test).

3.7. Effects of DEHP on StAR mRNA encoding steroidogenic acute regulatory protein

Testicular *StAR* mRNA in DEHP and E_2 treated goldfish remained unchanged following 7 d of exposure ($p > 0.05$; Fig. 7). Following 15 d of exposure, testicular *StAR* mRNA of DEHP treated goldfish showed no differences compared to control, while it was decreased in E_2 treated goldfish ($p < 0.05$). In both DEHP and E_2 treated goldfish, testicular *StAR* mRNA levels were decreased compared to the control following 30 d of exposure ($p < 0.05$).

3.8. Effects of DEHP on genes with products mediating estrogenic effects

Estrogenic activity was compared between DEHP and E_2 treated goldfish by studying *vtg* expression using a rabbit anti-sea bream vitellogenin polyclonal antibody cross-reacting with *Vtg* from cyprinids as well as evaluating *vtg* and *erα* mRNA levels in the liver and the brain *cyp19a1* mRNA levels (Fig. 8). Similar to control, western blotting showed no *Vtg* band in DEHP treated goldfish, while it was highly expressed in E_2 treated goldfish (Fig. 8A). At day 7 and 15, *cyp19a1b*, *erα*, and *vtg* mRNA levels remained unchanged in DEHP treated goldfish compared to the control ($p > 0.05$; Figs. 8B and S4). At day 30, *cyp19a1b* and *erα* mRNA levels were decreased in goldfish exposed to 1 μg/L DEHP ($p < 0.05$), however *vtg* mRNA levels were unchanged in DEHP treated goldfish ($p > 0.05$; Figs. 8B and S3). In E_2 treated goldfish, *cyp19a1b*, *erα* and *vtg* mRNA levels were increased compared to the control and DEHP treated goldfish at all time points tested ($p < 0.05$; Figs. 8B and S3). Testicular *cyp19a1a* and *erα* mRNA levels remained unchanged in both DEHP and E_2 treated goldfish ($p > 0.05$, data are not shown).

4. Discussion

The present study shows DEHP-reduced sperm quality in goldfish and illustrates DEHP effects on neuroendocrine and endocrine hormonal functions. The results indicate that DEHP has potency to decrease sperm production, motility and velocity in goldfish at environmentally relevant concentrations. To best of our knowledge, this is first study suggesting that DEHP-reduced sperm quality leading to decrease in male fertility might be due to inhibition of androgen production in fish. DEHP affects testicular hormonal functions by inhibiting LH production from pituitary functions or by disruption of cholesterol transfer to steroidogenesis pathway. However, DEHP does not affect the GnRH and Kiss-1/Gpr54 system.

Sperm production was decreased in DEHP treated goldfish, which is consistent with earlier studies on mammals (Lee et al., 2009; Vo et al., 2009; Pocar et al., 2012). However, GSI in DEHP treated goldfish was not different from that of the control, while it was significantly decreased in E_2 treated goldfish. This may suggest that testicular development was suppressed in E_2 treated goldfish (Maclatchy et al., 1997; Lahnsteiner et al., 2006 Lahnsteiner et al., 2006), but not in DEHP treated goldfish. The observed decrease in sperm production might be due to DEHP interfering with spermatogenesis (Uren-Webster et al., 2010; Corradetti et al., 2013). For the first time, we observed that DEHP reduces sperm motility

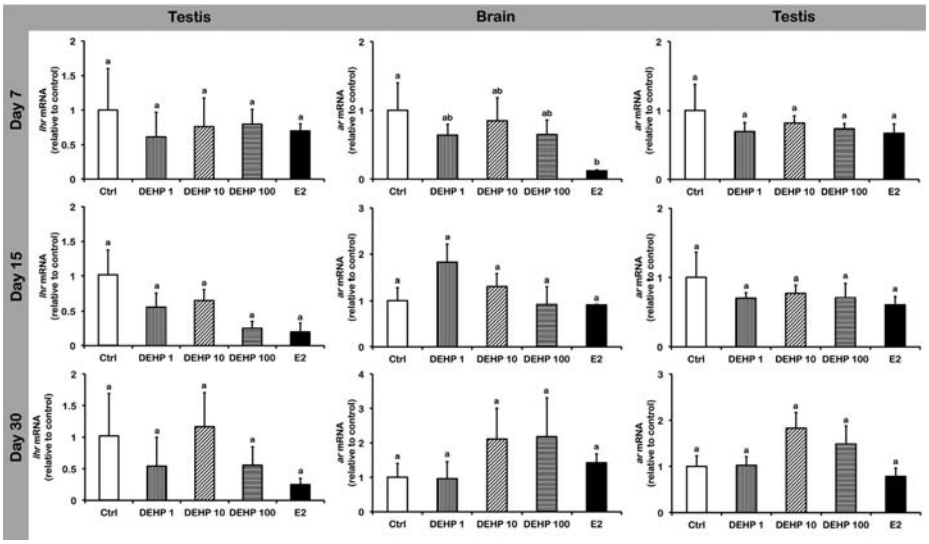

Fig. 6. mRNA levels of genes encoding luteinizing hormone receptor (*lhr*) and androgen receptor (*ar*) in the brain or testis of male goldfish exposed to di-(2-ethylhexyl)-phthalate (DEHP) and 17β-estradiol (E_2), *in vivo*. The brain and testis samples were collected from mature goldfish exposed to nominal 1, 10 or 100 μg/L DEHP and to 5 μg/L E_2 following 7, 15 and 30 d of exposure to study alternations in *lhr* and *ar* mRNA levels using a quantitative real-time PCR. Data were normalized to the internal control and are expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's *post hoc* test).

and velocity. This suggests that sperm maturation and the acquisition of sperm motility potential were disrupted in DEHP treated goldfish (Miura et al., 1992; Alavi et al., 2012b). Interestingly, the effects of DEHP on sperm motility and velocity were different. Soon after sperm motility initiation (15 s), sperm motility and velocity were decreased in goldfish exposed to DEHP. However, only sperm motility showed significant decreases in DEHP treated goldfish at 45 and 60 s post activation. These results suggest that DEHP mainly disrupts hormonal regulation of sperm maturation prerequisite to sperm motility potential, while sperm velocity depends on sperm intracellular pH, Ca^{2+}, and energy sources as well as molecular functions of proteins in the motility apparatus of the spermatozoa, the axoneme (Alavi et al., 2012a; Hatef et al., 2013).

To better understand DEHP-reduced sperm production, we measured 11-KT levels, which regulates fish spermatogenesis (Miura and Miura, 2003). 11-KT levels were decreased in DEHP treated goldfish following 15 and 30 d of exposure which is consistent with studies on mammals in which *in utero* or in prepubertal DEHP exposure decreased T levels in adults and male offspring (Parks et al., 2000; Akingbemi et al., 2001; Ge et al., 2007; Howdeshell et al., 2007; Culty et al., 2008; Lee et al., 2009; Hannas et al., 2012). This study suggests that DEHP is capable of inhibiting 11-KT production in fish following a chronic exposure; because 11-KT levels remained unchanged in DEHP treated goldfish following an acute (7 d) of exposure. Interestingly, 11-KT levels were lower in goldfish exposed to 1 μg/L DEHP compared to those of 10 and 100 μg/L at 30 d of exposure. Based on these finding, we infer that DEHP-induced inhibition of androgen production acts stronger at lower concentrations. It is worth to note that low dose effects of DEHP on reproductive endocrine system and male infertility have been previously suggested (Corradetti et al., 2013; Wang et al., 2013; Ye et al., 2014). In contrast to our results, previous studies have shown no changes (Crago and Klaper, 2012; Ye et al., 2014) or increases in T levels (Wang et al., 2013) in DEHP treated fish. These contradictory results of DEHP effects on androgen production in fish might be related to the different DEHP concentrations employed, duration of exposure, as well as the developmental stage in which the fish were exposed to DEHP. It is also worth noting that studying 11-KT levels is critical in fish since T acts as a substrate for E_2 and 11-KT synthesis (Young et al., 2005). In E_2 treated goldfish, 11-KT levels were decreased, confirming E_2-inhibited androgen production in fish (MacLatchy et al., 1997; Yamaguchi et al., 2006; Sharpe et al., 2007). Overall, these results suggest that DEHP-reduced sperm production is due to inhibition of 11-KT production in goldfish.

We observed that decreases 11-KT levels in DEHP treated goldfish following 15 d and 30 d of exposure were associated with decreases in circulatory LH levels. Our observation is consistent with studies on mammals, in which DEHP-inhibited LH release from the pituitary results in a decrease in T levels (Akingbemi et al., 2001; Culty et al., 2008). These findings suggest that DEHP acts on pituitary to inhibit testicular 11-KT production. In E_2 treated goldfish, LH production was inhibited following a chronic exposure associated with a decrease in 11-KT levels. Pituitary LH also regulates sperm maturation in fish (Miura and Miura, 2003; Alavi et al., 2012b). Observed decreases in sperm motility and velocity in DEHP treated goldfish following 30 d of exposure are also associated with a decrease in circulatory LH level. This suggests that DEHP-reduced sperm motility and velocity are due to inhibition of LH production and release from pituitary resulting in sperm maturation disruption.

No changes in *kiss1*, *gpr54* and *gnrh3* mRNA levels of goldfish exposed to DEHP observed in this study suggest that disruption of pituitary and testis hormonal functions is not mediated through DEHP effects on upstream neuroendocrine regulators. This is due to the fact that kisspeptin, through its receptor (*gpr54*), stimulates

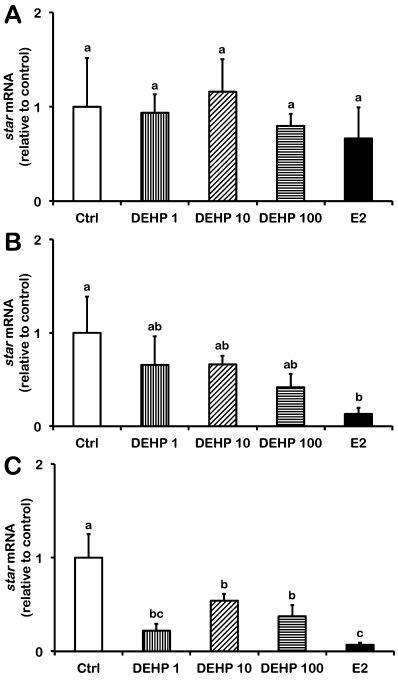

Fig. 7. mRNA levels of the gene encoding steroidogenic acute regulatory protein (*StAR*) in the testis of male goldfish exposed to di-(2-ethylhexyl)-phthalate (DEHP) and to 17β-estradiol (E_2), *in vivo*. The testis samples were collected from mature male goldfish exposed to nominal 1, 10 or 100 μg/L DEHP and to 5 μg/L E_2 following 7 (A), 15 (B) and 30 (C) d of exposure to study alternations in *StAR* mRNA levels using a quantitative real-time PCR. Data were normalized to the internal control and are expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's *post hoc* test).

GnRH and LH from hypothalamus and pituitary respectively (Li et al., 2009; Yang et al., 2010). In contrast, E_2 differentially acts on upstream neuroendocrine regulators of the pituitary and testicular functions. Following an acute exposure (7 d), *gnrh3* mRNA levels were decreased in E_2 treated goldfish suggesting (if the mRNA expression correlates with protein activity) that the hypothalamus is involved in inhibition of androgen production in E_2 treated goldfish. Following 15 and 30 d of exposure, inhibition of LH production and release from pituitary in E_2 treated goldfish leads to decrease in androgen production. In E_2 treated goldfish, *kiss1* and *gpr54* mRNAs were increased following 30 d of exposure, suggesting E_2 regulatory functions on the Kiss-1/GPR54 system, which has been shown in mammals (Smith et al., 2005; Clarkson et al., 2012). Overall, DEHP-reduced LH and androgen productions are not mediated through upstream neuroendocrine regulators.

Testicular *StAR* mRNA levels were decreased in DEHP treated goldfish following 30 d of exposure which is consistent with the former studies on male rats (Howdeshell et al., 2007; Hannas et al., 2012). These suggest that DEHP-inhibited androgen production might be due to the transfer of precursor to the steroidogenesis. In contrast, Ye et al. (2014) observed an increase in testicular *StAR* mRNA levels in marine medaka exposed to DEHP from hatching to the maturity stage, which does not conform with unchanged T levels. But in DEHP treated adult fathead minnow, testicular *StAR* mRNA levels and T levels remained unchanged (Crago and Klaper et al., 2012). The differences among these studies might be due to species-specific, and time- and dose-dependent effects of DEHP on *StAR* function in steroidogenesis. However, E_2 acts on testicular *StAR* mRNA levels which may affect sex steroid production *via* changes in the transfer of cholesterol to steroidogenesis pathway.

Our results show that neither *lhr*, *erα* nor *ar* changed in DEHP treated goldfish during the period of exposure, which is consistent with earlier studies on fish (Uren-Webster et al., 2010; Crago and Klaper, 2012) and mammals (Ge et al., 2007). These data suggest that DEHP-impaired reproduction is not mediated through hormonal receptors. In contrast, E_2 acts through receptor mediated pathways to affect neuroendocrine and endocrine regulation of reproduction (Burmeister et al., 2007; Marlatt et al., 2008; Liu et al., 2009; Nelson and Habibi, 2010).

In this study, we also examined aromatase activity of DEHP seen in fish (Wang et al., 2013; Ye et al., 2014) and mammals (Akingbemi et al., 2004; Andrade et al., 2006b) and compared with a group of goldfish exposed to E_2. E_2 and the E_2/11-KT ratio in blood plasma increased in E_2 treated goldfish associated with an increase in *cyp19a1b* mRNA, a gene encoding estrogen-responsive elements that converts androgens into estrogens in fish (Callard et al., 2001; Kishida et al., 2001). Vtg induction is associated with increases in *vtg* and *erα* mRNA levels in E_2 treated goldfish (Marlatt et al., 2008; Nelson and Habibi, 2010). In contrast, this study shows that all of these parameters remained unchanged or decreased in DEHP treated goldfish similar to fathead minnow (Crago and Klaper, 2012). In contrast, DEHP increased E_2 levels and the mRNA levels of genes encoding brain, liver or testicular *vtg* and er subtypes in mature rare minnow at 39.4 μg/L (Wang et al., 2013), in

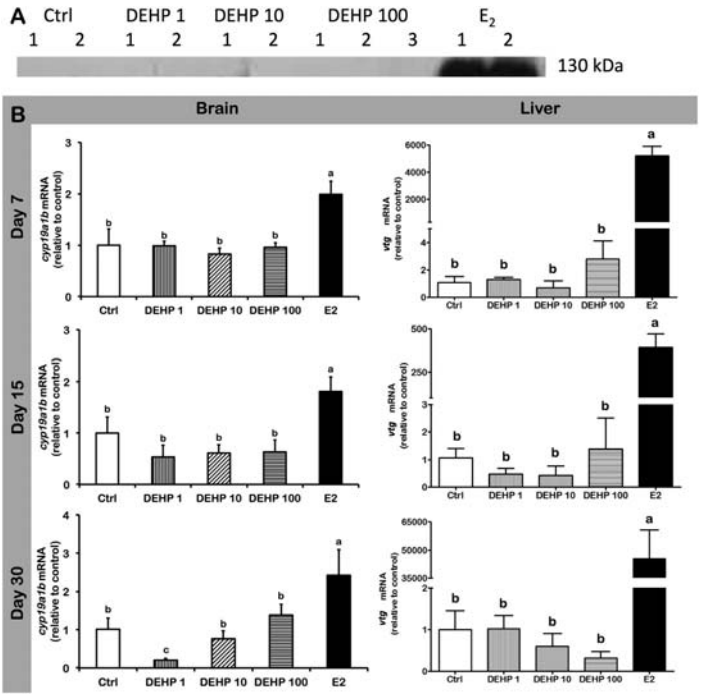

Fig. 8. Di-(2-ethylhexyl)-phthalate (DEHP) does not exhibit estrogenic activity. Expression of vitellogenin (*vtg*, A) and alternations in the mRNAs of the genes encoding brain cytochrome P450 aromatase (*cyp19a1b*, B) and the liver vitellogenin (*vtg*, B) of mature male goldfish were studied following exposure to nominal 1, 10 or 100 μg/L DEHP and to 17β-estradiol (5 μg/L, E_2), *in vivo*. For western blotting, induction of *Vtg* was shown in mature male goldfish exposed to E_2 following 30 d of exposure (A). The brain and liver samples were collected from mature male goldfish exposed to DEHP or E_2 to study alternations in *cyp19a1b* and *vtg* mRNA using a quantitative real-time PCR following 7, 15, and 30 d of exposure. Data were normalized to the internal control and are expressed as mean ± SEM ($n = 6$). Values with different superscripts are significantly different ($p < 0.05$, ANOVA with Tukey's *post hoc* test).

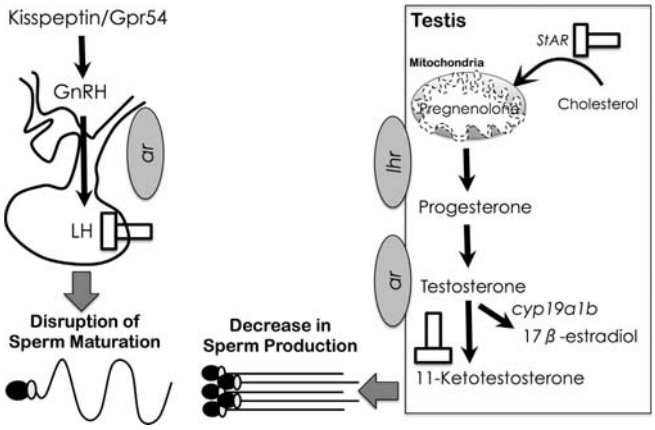

Fig. 9. A model representing neuroendocrine and endocrine disorders in goldfish exposed to di-(2-ethylhexyl)-phthalate (DEHP) that lead to diminished sperm quality. Following a chronic exposure (15–30 d), 11-KT levels were reduced in goldfish exposed to 1, 10 or 100 μg/L DEHP. At the same time, circulatory LH levels were decreased in DEHP treated goldfish. In addition, *StAR* mRNA levels were decreased in DEHP treated goldfish following 30 d of exposure. However, DEHP was without effects on *gnrh3*, *kiss1*, *kiss2* and *gpr54* mRNA levels. Overall, DEHP acts on pituitary and testicular hormonal functions to reduce sperm production, motility and velocity in goldfish.

marine medaka at 100 and 500 μg/L (Ye et al., 2014) or zebrafish at 5000 mg/kg b.w. (Uren-Webster et al., 2010). However, E_2/11-KT ratio and *cyp19a1b* mRNA decreased in both marine medaka and rare minnow. These studies suggest that DEHP estrogenic activity may differ among species. However, it does not exhibit estrogenic activity in zebrafish, fathead minnow, marine medaka and goldfish at the environmentally relevant concentrations in the polluted areas (below 100 μg/L).

In conclusion, the present study provides novel information on DEHP-related reproductive disorders in goldfish (Fig. 9). DEHP is capable of reducing sperm production, motility and velocity in goldfish following 30 d of exposure. Significant decreases in 11-KT and LH levels were observed following 15–30 d of exposure. These results suggest that DEHP-reduced sperm quality is due to DEHP effects of testicular and pituitary hormonal functions. However, DEHP does not affect GnRH and Kiss-1/Gpr54 systems to alter pituitary and testicular hormonal functions. In addition, DEHP does not exhibit estrogenic activity and does not act through sex steroid receptor.

Conflict of interest

The authors have declared that no competing interests exist.

Supporting information

Additional data is shown in Table S1 and Fig. S1–S3.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2015.03.017.

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CHAPTER 4

GENERAL DISCUSSION

The present thesis shows that VZ and DEHP effects on male fertility in fish is due to disruption of pituitary and testicular hormonal functions associated with alternations in transcripts mRNA levels of genes encoding enzymes in steroidogenesis. For the first time, we have studied the effects of ECs on Kiss/Gpr54 system, which plays key role in regulation of hypothalamus and pituitary hormonal functions. We established a combination exposure protocol in which goldfish were exposed to experiment endogenous sex steroids as positive/negative controls to better understand VZ and DEHP effects on reproductive endocrine system in fish.

4.1. Sperm production, motility and velocity were affected by vinclozolin and di-(2 ethylhexyl)-phthalate

Since male fertility is highly depend on sperm quality, therefore evaluation of sperm motility kinetics is a suitable biomarker to better understand the impacts of environmental contaminants on male fertility (McAllister and Kime, 2003; Lahnsteiner et al., 2006). We observed that sperm production was decreased in goldfish exposed to DEHP (Golshan et al., 2015) and VZ (Hatef et al., 2012). However, GSI was not differed in DEHP and VZ-treated goldfish and was significantly decreased in E_2-treated goldfish compared to control. This may suggest that testicular development was suppressed in E_2-treated goldfish (Maclatchy et al., 1997; Lahnsteiner et al., 2006), but not in DEHP and VZ-treated goldfish. The observed decrease in sperm production in goldfish exposed to VZ and DEHP might be due to disruption in spermatozoa production during spermatogenesis (Uren-Webster et al., 2010; Corradetti et al., 2013).

This is first report showing that sperm motility and velocity were decreased in goldfish exposed to DEHP (Golshan et al., 2015) and VZ (Hatef et al., 2012). These results provide valuable reason for observed decrease in fertility of fathead minnow exposed to VZ (Martinovic et al., 2008), zebrafish and marine medaka exposed to DEHP (Uren-Webster et al., 2010; Corradetti et al., 2013; Ye et al., 2014). We could not collect sperm from E_2-treated goldfish; however it has shown that sperm motility and velocity were decreased in salmonids exposed to E_2 for 35-50 days (Lahnsteiner et al., 2006). These suggest that sperm maturation and the acquisition of sperm motility potential were disrupted in VZ and DEHP-treated goldfish. The effects of VZ and DEHP on sperm motility and velocity were dose-dependent and could be related to the multifunctional potencies of VZ and DEHP to interfere with hormonal regulation of sperm maturation or with intracellular signal transductions required for the acquisition of sperm motility potential (Miura et al., 1992; Alavi et al., 2012a,b). In this study, we did not study sperm energetic and metabolism; however it is also possible that VZ, DEHP or E_2-reduced sperm motility and velocity which could be related to sperm ATP content and metabolism of energy (Hatef et al., 2011, 2013).

4.2. Testicular hormonal functions were affected by vinclozolin and di-(2-ethylhexyl)-phthalate

To better understand VZ and DEHP-reduced sperm quality, 17β-estradiol and 11-KT levels which regulate initiation and maintenance of spermatogenesis (Miura and Miura, 2003) were measured. The effects of VZ and DEHP on androgen production were different. In VZ -treated goldfish, a biphasic effect on 11-KT production was observed. Following a chronic exposure to low dose of VZ (100 μg/L), 11-KT levels were increased, while it was decreased in goldfish exposed to high concentration (800 μg/L) following both acute and chronic exposure. In

mammals, VZ-induced increase in T production has been reported in male rats (Kubota et al., 2003; Loutchanwoot et al., 2008; Quignot et al., 2012), while in fish VZ caused no change in 11-KT production in fathead minnow exposed for 21 days (Makynen et al., 2000; Martinovic et al., 2008). 17β-estradiol levels remained unchanged in goldfish exposed to VZ, however E_2/11-KT ratio were increased and decreased in goldfish exposed to high (800 μg/L) and low (100 μg/L) dose of VZ. These results suggest that VZ at low dose is capable of altering 11-KT production in goldfish depending on duration of exposure which resulting in imbalance E_2/11-KT ratio.

In contrast to VZ, 11-KT levels were decreased in DEHP-treated goldfish following 15 and 30 d of exposure which is consistent with studies on mammals in which *in utero* or in prepubertal DEHP exposure decreased T levels in adults and male offspring (Parks et al., 2000; Akingbemi et al., 2001; Ge et al., 2007; Howdeshell et al., 2007; Culty et al., 2008; Lee et al., 2009; Hannas et al., 2012). This study suggests that DEHP is capable of inhibiting 11-KT production in fish following a chronic exposure. In contrast to our results, previous studies have shown no changes (Crago and Klaper, 2012; Ye et al., 2014) or increases in T levels (Wang et al., 2013) in DEHP-treated fish. We observed no changes in E_2 levels in goldfish exposed to DEHP which consistent with previous study on fathead minnow (Crago and Klaper, 2012).

In E_2-treated fish, the 11-KT level decreased following either acute or chronic exposure indicating that E_2 impairs androgen synthesis in fish (Yamaguchi et al., 2006; Sharpe et al., 2007), while estradiol increased sharply following acute and chronic exposure.

Taken together, our results show that decrease in sperm production in fish exposed to VZ and DEHP might be related to disruption of E_2/11-KT balance and androgen production which are critical for spermatogenesis and sperm production.

4.3. Neuroendocrine hormonal functions were affected by vinclozolin and di-(2-ethylhexyl)-phthalate

At maturity stage, the pituitary LH is the primary regulator of androgen biosynthesis in the testis (Nagahama, 1994). We observed that LH levels were increased in goldfish exposed to low dose of VZ (100 μg/L) following a chronic exposure which is consistent with increase in 11-KT levels at the same period of exposure. This indicates that stimulation of 11-KT production in goldfish exposed to VZ is mediated by alteration in pituitary hormonal function and suggests that the negative feedback of androgen to control LH release from pituitary was disrupted (Van der Kraak, 2009). Similarly, VZ-induced androgen production mediated by LH has also been observed in male rats (O'Connor et al., 2002; Kubota et al., 2003; Loutchanwoot et al., 2008). In fathead minnow demonstrated no changes in *lh* mRNA level following exposure to VZ (Villeneuve et al., 2007).

In DEHP-treated goldfish, LH levels were decreased which was in consistence with decrease in 11-KT levels. Similar results were observed in mammals where DEHP-inhibited LH release from the pituitary results in a decrease in T levels (Akingbemi et al., 2001; Culty et al., 2008). These findings suggest that DEHP acts on pituitary to inhibit testicular 11-KT production.

Overall, in both DEHP and VZ-treated goldfish, alternations in androgen production were associated with disruption of pituitary secretion of LH. Since LH regulates sperm maturation and acquisition of the sperm potential for motility in fish through stimulating testicular hormone (Miura and Miura, 2003; Alavi et al., 2012b), these results suggest that VZ and DEHP-decreased sperm motility and velocity in goldfish might be due to disruption pituitary LH.

In this study, we investigated upstream neuroendocrine regulators of LH in fish exposed to ECs. Results showed that *gnrh3* mRNA levels were decreased and increased in goldfish exposed to VZ following an acute and chronic exposure, respectively which were consistent with 11-KT levels (Golshan et al., 2014). In addition, *kiss1* and *gpr54* mRNA levels were increased in goldfish exposed to VZ similar to that of flutamide treated goldfish (Golshan et al., unpublished data). In contrast, no changes in *kiss1*, *gpr54* and *gnrh3* mRNA levels were observed in DEHP-treated goldfish. As GnRH and Kiss-1/Gpr54 system regulate pituitary and testicular hormonal functions (Van der Kraak, 2009), these results suggest that VZ and DEHP differentially act on upstream neuroendocrine regulators of pituitary and testicular hormonal functions to impair spermatogenesis and sperm quality in goldfish. In contrast to DEHP, VZ is capable of interfering with Kiss-1/Gpr54 system and hypothalamus hormonal function to affect spermatogenesis and sperm maturation. In E_2-treated goldfish, *gnrh3* mRNA levels were decreased following an acute exposure suggesting that the hypothalamus is involved in inhibition of androgen production. Also, in E_2-treated goldfish, *kiss1* and *gpr54* mRNA were increased after chronic exposure, suggesting E_2 regulatory functions on the Kiss-1/Gpr54 system, which has been shown in mammals (Smith et al., 2005; Clarkson et al., 2012).

Taken together, VZ-stimulated and DEHP-inhibited 11-KT production is mediated by their effects on pituitary hormonal functions. However it is only VZ that shows capability to interfere with hypothalamus hormonal function through alternations in Kiss-1/Gpr54 system.

4.4. Effects of vinclozolin and di-(2-ethylhexyl)-phthalate on steroidogenesis

Since DEHP and VZ are capable of interfering with 11-KT production, we studied substrate availability for sex steroid biosynthesis and alternations in mRNA levels of selected genes that encode enzymes in steroidogenesis pathway (Golshan et al., 2014, 2015). The results show that total cholesterol, HDL and LDL remained unchanged in VZ-tretaed goldfish. This suggests that alternation in 11-KT biosynthesis in goldfish exposed to VZ could not be related to precursor availability (Sharpe et al., 2007). However, *star*, *cyp450, 3ϐhsd* and *cyp17* mRNA levels were increased in goldfish exposed to VZ. In contrast, in fish exposed to DEHP, testicular *star* mRNA levels were decreased following chronic exposure. These results are consistent with increase and decrease in 11-KT levels in VZ and DEHP-treated goldfish suggesting that VZ and DEHP-induced testicular hormonal function might be due to disruption of the transfer of precursor to the steroidogenesis or disruption of genes that encodes enzymes in steroidogenesis pathway. In E_2-treated fish, mRNA of steroidogenic enzyme genes decreased following both acute and chronic exposure, indicating that the decrease in 11-KT was also due to disruption in testicular function (Sharpe et al., 2007).

4.5. Effects of vinclozolin and di-(2-ethylhexyl)-phthalate through androgen receptor

As 11-KT production was altered in goldfish exposed to VZ and DEHP, we studied *ar* mRNA levels in the brain and testis to understand whether their effects are mediated through a receptor mediated pathway. The brain *ar* mRNA levels were decreased in goldfish exposed to low dose VZ (100 µg/L) following an acute exposure, while it was increased following a chronic exposure. These alternations were associated consistent with changes in plasma 11-KT levels following an acute and chronic exposure, suggesting that VZ interfere with negative feedback of androgen at low dose to stimulate 11-KT production in the testis. It is worth noting that it has been suggested changing in *ar* mRNA correlate with the levels of sex steroids (Burmeister et al., 2007; Liu et al., 2009). However, *ar* mRNA levels remained unchanged in the testis of goldfish exposed to VZ. These results suggest that the brain AR might mediate VZ-related

disruption in 11-KT biosynthesis. In DEHP-treated goldfish, the brain and testicular *ar* mRNA levels remained unchanged, which is consistent with earlier studies in fish (Uren-Webster et al., 2010; Crago and Klaper, 2012) and mammals (Ge et al., 2007). These results suggest that DEHP-impaired reproduction is not mediated through hormonal receptors.

4.6. Estrogenic activity of vinclozolin and di-(2-ethylhexyl)-phthalate

We examined aromatase activity of DEHP, VZ and compared with a group of goldfish exposed to endogenous estrogen hormone (E_2). In E_2-treated goldfish, E_2 levels and E_2/11-KT ratio were increased associated with increases in *cyp19a1b*, *vtg* and *er* subtype mRNA levels. These results clearly showed E_2-treated goldfish induced estrogenic activity. Almost these parameters remained unchanged or decreased in VZ and DEHP-treated goldfish which consistent with previous studies (Martinovic et al., 2008; Crago and Klaper, 2012). There are some studies that suggest estrogenic activity of DEHP in rare minnow (Wang et al., 2013), marine medaka (Ye et al., 2014). However, E_2/11-KT ratio and *cyp19a1b* mRNA are decreased in these species and these alterations do not consist with observed increase in E_2 levels and the mRNA levels of genes encoding brain, liver or testicular *vtg* and *er* sub-types. These studies suggest that DEHP estrogenic activity may differ among species. The present studies suggest that VZ and DEHP effects on reproductive endocrine system are not mediated through stimulation of estrogenic activity in goldfish.

4.7. Conclusion and remarks

In conclusion, the present study provides novel information on the mechanisms through which VZ and DEHP disrupt neuroendocrine and endocrine hormonal functions that lead to diminsihed sperm quality (Figure 1). Both VZ and DEHP have potency to interfere with reproductive pathway and thus impair male fertility. Interestingly, VZ and DEHP effected differentially on testicular androgen production. At low dose (< 100 μg/L), VZ and DEHP stimulate and inhibit 11-KT production, respectively. Their effects on testicular hormonal function highly depend on the concentration and duration of exposure and mediated by alternation in pituitary hormonal functions. A combination protocol suggest an anti-androgenic activity of VZ similar to that of flutamide through the brain AR. However, observed inhibition of 11-KT in DEHP-treated goldfish may also suggest DEHP ani-androgenic activity (Luccio-Camelo and Prins, 2011), but it needs further investigation in a combination experiments using pure anti-androgen and androgen. In contrast to E_2, neither VZ nor DEHP exhibit estrogenic activity. The outcome of alternations in hypothalmous-pituitary-testis hormonal functions are to decrease sperm qualtiy and affect male fertility.

In aquatic environment, there are mixtures of environmental contaminants that may exhibit pharmacodynamic interactions. These interactions can be antagonistic or synergistic, which may cause as increase in toxicity of a particular chemical which is known as a cocktail effect. For example, co-exposure of ethinylestradiol and ketoconazole increased mRNA transcript of VTG nine times more than ethinylestradiol alone (Hasselberg, et al., 2008).

In this study, we observed that VZ and DEHP affected testicular functions. However the mechanisms through which they ineterfer with hormonal regulation of sperm maturation and sperm motility signaling are largely unknown. Future studies should investigate progestin-dependent sperm maturation, sperm energetics and metabolism and the effects of these chemicals on molecular function of sperm motility apparatus and sperm axoneme (Thomas et al., 1997; Peknicova et al., 2002; Tubbs et al., 2009; Tan et al., 2014; Cosson, 2010; Alavi et al., 2012a; Hatef et al., 2013).

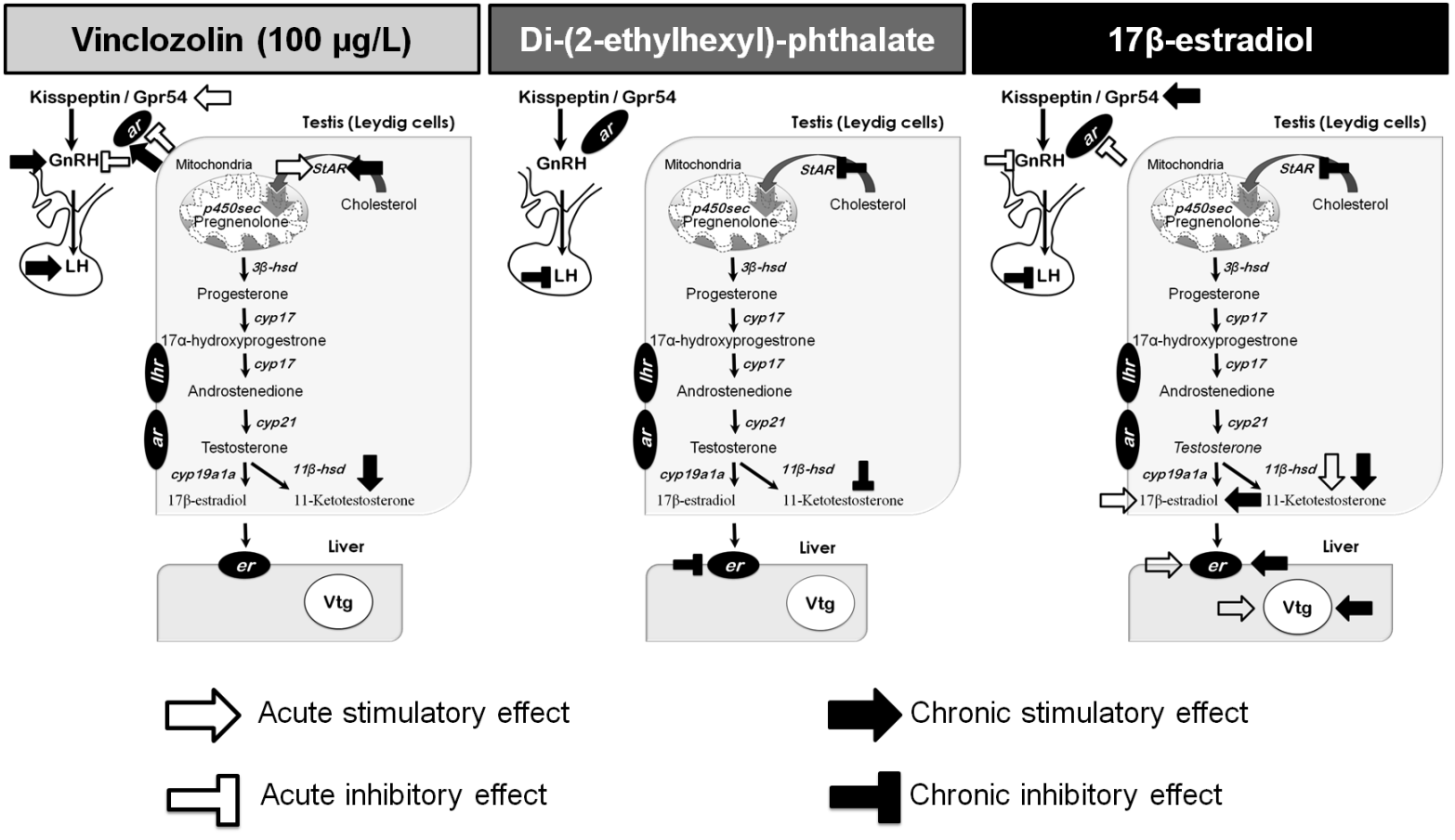

Figure 1. *A model representing neuroendocrine and endocrine disorders in goldfish exposed to VZ, DEHP and* E_2.

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ENGLISH SUMMARY

Environmental contaminants and endocrine associated male infertility in fish

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There are a large number of natural and synthetic environmental contaminants (ECs) known or suspected to mimic or interfere with male reproductive endocrine functions. Our current knowledge is largely addressed to ECs that induce oestrogen-induced feminization. However, there are several ECs that cause alternations in androgen production similar to oestrogenic ECs, but they do not induce vitellogenin-induced feminization. The mechanisms of action of these chemicals are still unclear due to the fact that androgen receptor (AR) functions in androgen-mediated male reproductive physiology are largely unknown.

In the present study, we examined the effects of vinclozolin (VZ) (100, 400 and 800 μg/L) and di-(2-ethylhexyl)-phthalate (DEHP) (1, 10 and 100 μg/L) on male reproductive performance in goldfish following 30 days of exposure. Since both VZ and DEHP act as agonists and/or antagonists of hormonal receptors, estrogen receptor subtypes (*er*) and androgen receptor (*ar)* mRNA levels were studied. For studying their oestrogenic activity, one group of goldfish were exposed to 17β-estradiol (E_2).

In DEHP-treated goldfish, sperm production, motility and velocity were decreased at 1, 100 and 10 μg/L, respectively. Our previous study has shown that sperm production, motility and velocity were decreased in goldfish exposed to 800 μg/L VZ. These suggest that DEHP and VZ are capable of interfering with spermatogenesis and sperm maturation. In E_2 treated goldfish, none of the males produced sperm, indicating E_2-suppressed spermatogenesis.

Similar to E_2-treated goldfish, 11-KT levels were decreased in goldfish exposed to ≥10 and ≥1 μg/L DEHP at day 15 and 30, respectively. In VZ-treated goldfish, 11-KT levels were decreased in goldfish exposed to 800 μg/L VZ following 7 d, but increased in goldfish exposed to 100 μg/L VZ following 30 d of exposure. E_2 levels remained unchanged and increased in DEHP and E_2-treated goldfish, respectively.

In VZ treated goldfish, LH levels were increased at 100 μg/L. In contrast, LH levels were decreased in DEHP and E_2-treated goldfish following 15 d of exposure. There were also positive relationships between LH and 11-KT levels suggesting that inhibition or stimulation of androgen production were mediated by DEHP or VZ-induced alternations in pituitary function.

In VZ-treated goldfish, *gnrh3* mRNA levels were decreased following 7 d of exposure and increased at low dose following 30 d of exposure. *kiss1* or *kiss2* mRNA levels were also increased in VZ-treated goldfish, while *gpr54* mRNA remained unchanged. In DEHP-treated goldfish, *gnrh3*, *kiss1* and its receptor (*gpr54*) mRNA levels did not change during the period of exposure. In E_2-treated goldfish, *gnrh3* mRNA levels were decreased at day 7, but *kiss1* and *gpr54* mRNA levels were increased at day 30 of exposure. These results suggest that, in contrast to DEHP, VZ effects on pituitary and testicular functions are mediated by disruption of hypothalamus function and upstream neuroendocrine regulators.

The brain *ar* and testicular *lhr* mRNA levels were changed in VZ and E_2-treated goldfish depending on dose and period of treatment, however they remained unchanged in DEHP-treated goldfish. These differences suggest that DEHP may act through an independent hormonal receptor pathway, while VZ acts through a receptor pathway.

vtg, er and cyp19a1b remained unchanged in DEHP and VZ-treated goldfish but increased in E2-treated goldfish. These indicate that neither VZ nor DEHP acts as oestrogenic compound to impair male fertility.

In conclusion, DEHP and VZ-reduced sperm quality in goldfish due to stimulation and inhibition of 11-KT production which were mediated by alternations in pituitary function to produce LH or by disruption of the transfer of cholesterol to steroidogenesis. Upstream neuroendocrine regulators (*gnrh3* and *kiss-1/gpr54*) were disrupted in VZ-treated goldfish. Taken together, VZ and DEHP differentially act on brain and testis to impair fertility endpoints.

CZECH SUMMARY

Znečištění životního prostředí a endokrinně podmíněná neplodnost samců ryb

Mahdi Golshan

V životním prostředí se vyskytuje velké množství přírodních a syntetických kontaminantů (ECs), u kterých je známo nebo existuje podezření, že napodobují nebo interferují s endokrinními funkcemi u samčí reprodukce. Naše dosavadní poznatky ukazují, že právě tyto kontaminanty mohou způsobit estrogeny podmíněnou feminizaci. Nicméně existuje několik ECs, které podobně jako estrogenní ECs způsobují změny v produkci androgenů, ale nezpůsobují vitelogeninem podmíněnou feminizaci. Mechanizmy působení těchto látek jsou stále nejasné, vzhledem k tomu, že jsou do značné míry neznámé funkce androgenního receptoru (AR) ve fyziologii androgenně-zprostředkované samčí reprodukce.

V této studii jsme zkoumali účinky vinklozolinu (VZ) (100, 400 a 800 μg/L) a di-(2-ethylhexyl) ftalátu (DEHP) (1, 10 a 100 μg/L) na reprodukci samců karasů po dobu 30 dnů. Vzhledem k tomu, že VZ i DEHP působí jako agonisté a/nebo antagonisté hormonálních receptorů, byly studovány subtypy estrogenů (*er*) a hladiny mRNA androgenních receptorů (*ar*). Kvůli studiu estrogenní aktivity byla jedna skupina ryb vystavena působení 17β-estradiolu (E_2).

U ryb vystavených DEHP (1, 10 a 100 μg/L) došlo ke snížení produkce spermií, pohyblivosti a rychlosti. Naše předchozí studie ukázaly, že produkce spermií, pohyblivost a rychlost se u ryb snižují při expozici VZ o koncentraci 800 μg/L. To naznačuje, že DEHP a VZ jsou schopny zasahovat do spermatogeneze a dozrávání spermií. Žádný ze samců vystavených účinku E_2 neprodukoval spermie, což ukazuje na potlačenou spermatogenezi.

Podobně jako u ryb exponovaných E_2, také u ryb vystavených koncentracím DEHP ≥10 a ≥1 μg/L bylo pozorováno snížení hladiny 11-KT, a to konkrétně v 15. a 30. dnu testování. U ryb exponovaných VZ bylo zaznamenáno snížení hladiny 11-KT, a to u ryb vystavených koncentraci 800 μg/L VZ po dobu 7 dní, naproti tomu jsme zaznamenali její zvýšení u ryb vystavených koncentraci 100 μg/ VZ po dobu 30 dnů. Hladiny E_2 zůstaly nezměněny u ryb vystavených DEHP a zvýšení došlo u skupiny, kde byl použit E_2.

Hladiny LH se zvýšily u ryb vystavených koncentraci VZ 100 μg/L. Na rozdíl od ryb vystavených účinkům DEHP a E_2, kde byly hladiny LH po 15 dnech expozice sníženy. Zde byla také zjištěna souvislost mezi hladinami LH a 11-KT, což vypovídá, že inhibice nebo stimulace produkce androgenu byla způsobena změnami ve funkci hypofýzy vyvolanými právě působením DEHP nebo VZ.

U ryb vystavených VZ se snižovaly hladiny *gnrh*3 mRNA po dobu 7denní expozice a zvyšovaly se při nízkých dávkách během 30denní expozice. *kiss1* nebo *kiss2* mRNA hladiny také vzrostly, zatímco *gpr54* mRNA zůstala nezměněna. U ryb vystavených působení DEHP se hladiny *gnrh3*, *kiss1* a jeho receptoru (*gpr54*) mRNA během doby expozice nezměnily. U ryb exponovaných E_2 byly hladiny *gnrh3* mRNA sníženy 7. den působení, ale hladiny *kiss1* a *gpr54* mRNA byly zvýšeny v 30. den expozice. Tyto výsledky ukazují, že na rozdíl od DEHP, VZ narušuje funkce hypotalamu a zpětnovazebné mechanizmy v neuroendokrinní regulaci, což ovlivňuje činnost hypofýzy a testikulární funkce.

Hladiny mRNA *ar* v mozku a *lhr* v testes byly ovlivněny v závislosti na dávce a době působení VZ a E_2, ale zůstaly nezměněny u ryb vystavených DEHP. Tyto rozdíly vypovídají o tom, že DEHP může působit přes nezávislou hormonální receptorovou cestu, zatímco VZ působí přes receptorovou cestu.

Vtg, *er* a *cyp19a1b* zůstaly beze změny u ryb vystavených DEHP a VZ, ale vzrostly u ryb vystavených účinkům E_2. Ani VZ, ani DEHP tedy nepůsobí jako estrogenní kontaminanty narušující samčí plodnost.

Výsledkem naší práce bylo zjištění, že v důsledku stimulace nebo inhibice produkce 11-KT prostřednictvím VZ a DEHP se snižuje kvalita spermií ryb. K tomuto dochází z důvodu narušení funkce hypofýzy produkovat LH nebo narušením přenosu cholesterolu pro steroidogenezi. U ryb vystavených VZ byly přerušeny zpětněvazebné mechanizmy neuroendokrinních regulátorů (*gnrh3* a *kiss-1/gpr54*). Společné působení VZ a DEHP ovlivňuje funkce mozku a testes a má negativní dopad na samčí plodnost.

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LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Golshan, M., Habibi, H.R., Alavi, S.M.H., 2015. Alterations in transcripts of neuroendocrine reproductive genes in goldfish exposed to anti-androgens, testosterone and their combinations. Manuscript.

Golshan, M., Hatef, A., Socha, M., Milla, S., Butts, I.A.E., Carnevali, O., Rodina, M., Sokolowska-Mikolajczyk, M., Fontaine, P., Linhart, O., Alavi, S.M.H., 2015. Di-(2-ethylhexyl)-phthalate disrupts pituitary and testicular hormonal functions to reduce sperm quality in mature goldfish. Aquat. Toxicol. 163, 16–26.

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Hatef, A., Alavi, S.M.H., **Golshan, M.**, Linhart, O., 2013. Toxicity of environmental contaminants to fish spermatozoa function *in vitro*-A review. Aquat. Toxicol. 140–141: 134–144.

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

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Golshan, M., Hatef, A., Fontaine, P., Habibi, H.R., Alavi, S.M.H., 2013. Reproductive neuroendocrine disruption in male goldfish exposed to Vinclozolin. Annual meeting of the Society for the Study of Reproduction (SSR). Montreal, Canada. July 21–27.

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Golshan, M., 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. In: SETAC CEE 3rd Annual Meeting, Krakow. Poland. September 16–19.

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AQUA 2012 – Global Aquaculture – Securing Our Future. September 1–5, Prague, Czech republic (*Poster presentation*).	2012
SETAC CEE 3rd Annual Meeting, September 16–19, Krakow, Poland (*Poster presentation*).	2012
Fish and amphibian embryos as alternative models in toxicology and teratology, Aunaly-sous-Bios. October 11–12, Paris, France (*Poster presentation*).	2012
Annual meeting of the Society for the Study of Reproduction (SSR). July 21–27, Montreal, Canada (*Poster presentation*).	2013
Diversification in Inland Finfish Aquaculture II (DIFA II). September 24–26, Vodňany, Czech Republic (*Oral presentation*).	2013
10th International Symposium on Reproductive Physiology of Fish. May 25–30, Olhao, Portugal (*Oral presentation*).	2014

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Prof. Hamid. R Habibi, The University of Calgary, Alberta, Canada (1.5 month, qRT-PCR)	2012
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Grant Agency of the University of South Bohemia 067/2013/Z
Modulations in neuroendocrine regulation of steroidogenesis in male goldfish exposed to anti-androgen vinclozolin. (leader Mahdi Golshan)

AWARDS

2010 – Honour student, Department of Fisheries and Environmental Sciences, the University of Tehran.
2013 – Elsevier Distinguished Article – The most downloaded article
Hatef, A., Alavi, S.M.H., Golshan, M., Linhart, O., 2013. Toxicity of environmental contaminants to fish spermatozoa functions *in vitro* – A review. Aquat. Toxicol. 140–141, 134–144.

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

2012 – the University of Calgary, Alberta, Canada, Lab of Prof. Hamid. R Habibi. qRT-PCR (1.5 month).
2013 – the University of Calgary, Alberta, Canada, Lab of Prof. Hamid. R Habibi. Digital PCR (4 months).

PH.D. COURSES

Basics of scientific communication
Pond aquaculture
Applied hydrobiology
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