Effects of exposures to the plasticiser, di-n-butyl phthalate and the pharmaceutical, flutamide on the biomarkers of reproduction in Australian freshwater fish species, Murray rainbowfish (*Melanotaenia fluviatilis*)

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To

Mumma and Papa
Every bit of me, is a little bit of you

and

My husband
For making me wonder who I am

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DECLARATION

I certify that this work contains no material which has been accepted for the award of any degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide.

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1. Harpreet Bhatia, Anupama Kumar, Yukiko Ogino, Jun Du, Adrienne Gregg, John Chapman, Mike J. McLaughlin and Taisen Iguchi (2014) Effects of the commercial anti-androgen flutamide on the biomarkers of reproduction in male Murray rainbowfish (*Melanotaenia fluviatilis*). Environmental Toxicology and Chemistry 33(5): 1098 – 1107.

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3. Harpreet Bhatia, Anupama Kumar, Yukiko Ogino, Adrienne Gregg, John Chapman, Mike J. McLaughlin and Taisen Iguchi (2014) Di-n-butyl phthalate causes estrogenic effects in adult male Murray rainbowfish (*Melanotaenia fluviatilis*). Aquatic Toxicology 149: 103 – 115.
4. Harpreet Bhatia, Anupama Kumar, Jun Du, John Chapman and Mike J. McLaughlin (2013) Di-n-butyl phthalate causes anti-estrogenic effects in female Murray rainbowfish (*Melanotaenia fluviatilis*). Environmental Toxicology and Chemistry 32(10): 2335 – 2344.
5. Harpreet Bhatia, Anupama Kumar, John Chapman, Mike J. McLaughlin (In press) Long-term exposures to di-n-butyl phthalate inhibit body growth and impair gonad development in juvenile Murray rainbowfish (*Melanotaenia fluviatilis*). Journal of Applied Toxicology. DOI 10.1002/jat.3076 (Accepted August 30, 2014).
6. Harpreet Bhatia, Anupama Kumar, Jun Du, John Chapman, Mike J. McLaughlin. Effects of the model anti-androgen, flutamide on 17β-estradiol-induced hormonal imbalance in freshwater juvenile Murray rainbowfish (*Melanotaenia fluviatilis*) (In the process of submission).

HARPREET BHATIA **DATE**

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Di-n-butyl phthalate; E2:17β-Estradiol; VTG: Vitellogenin; 11-KT: 11-Ketosterone; CF: Condition factor

LIST OF ABBREVIATIONS

Acox-1	Acyl-coenzyme A oxidase 1
AChE	Acetyl choline esterase
ACP	Acid phosphatase
AGD	Ano-genital distance
ALP	Alkaline phosphatase
AMH	Anti-mullerian hormone
ANOVA	One-way analysis of variance
AR	Androgen receptor
Ad4BP/SF-1	Adrenal 4 binding protein/steroidogenic factor 1
AST	Aspartate transaminase
BBP	Butyl benzyl phthalate
BFTSA	N,O-bis(trimethylsilyl)-trifluoroacetamide
BMP	Bone morphogenetic protein
BPA	Bisphenol A
BW	Body weight
CALUX	Chemically activated luciferase gene expression
CAT	Catalase
CF	Condition factor
ChG	Choriogenin
CYP3A4	cytochrome P4503A4
DBBzP	Dibutyl benzyl phthalate
DCHP	Dicyclohexyl phthalate
DDT	Dichlorodiphenyltrichloroethane
DDE	Dichlorodiphenyldichloroethylene
DEHP	Diethyl hexyl phthalate
DEP	Diethyl phthalate
DMP	Dimethyl phthalate
Dmrt1	doublesex/mab-3 related transcription factor
DnBP	Di-n-butyl phthalate
DOP	Dioctyl phthalate
dph	Days post hatch
DiNP	Di-iso-nonyl phthalate
DO	Dissolved oxygen
DPP	Dipropyl phthalate
E1	Estrone
E2	17β-Estradiol
EDC	Endocrine disrupting chemical
EE2	Ethynyl estradiol
ehhadh	Hydratase/3-hydroxyacyl coenzyme A dehydrogenase
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EMB	Embryonic
ER	Estrogen receptor
ERE	Estrogen-reponse element
EROD	Ethoxyresorufin-o-deethylase
FEQ	Flutamide equivalent
FSH	Follicle stimulating hormone
GC-MS	Gas chromatography – mass spectroscopy

GD	Gestation day
GLUT	Facilitative glucose transporter
GHR	Growth hormone receptor
GPx	Glutathione peroxidase
GR	Glutathione reductase
GH	Growth hormone
GHR	Growth hormone receptor
GVBD	Germinal vesicle breakdown
GSH	Glutathione
GSI	Gonadosomatic index
GST	Glutathione S-transferase
HPG	Hypothalamo-pitutary-gonadal
HPLC	High pressure liquid chromatography
HSI	Hepatosomatic index
INSL3	Insulin like peptide 3
IP	Intra-peritoneal
LAC	Lactational
LDH	Lactate dehydrogenase
IGF-1R	insulin-like growth factor-I receptor
LHR	Luteinising hormone receptor
lpl	Lipoprotein lipase
LOD	Limit of detection
LOEC	Lowest observed effect concentration
LOQ	Limit of quantification
LPO	Lipid peroxidase
MBP	Mono butyl phthalate
MBzP	Monobenzyl phthalate
MEHP	Methyl hexyl phthalate
MEP	Mono ethyl phthalte
MEHHP	Mono-(2-ethyl-5-hydroxyhexyl) phthalate
MEOHP	Mono-(2-ethyl-5-oxohexyl) phthalate
M1	2-[(3,5-dichlorophenyl)-carbamoyl]oxy-2-methyl-3-butenoic acid
M2	3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide
MT	17α-Methyl testosterone
MoA	Mode of action
MS222	Methane tricainesulfonate
Nrc1	HR-associated Cell death 1
PAE	Phthalic acid ester
PBS	Phosphate buffer saline
PND	Post-natal development
Ptgs2	Prostaglandin-endoperoxide synthase 2
P450c17	Cytochrome P450, family 17, subfamily A, polypeptide 1
P450scc	Cholesterol side chain cleavage enzyme
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator-response element
PR	Progesterone receptor
Sox9	Sex determining region Y-box 9
SR-B1	Scavenger receptor class B type 1
StAR	Steroid acute regulatory protein
STP	Sewage treatment plant

T	Testosterone
THR	Thyroid hormone receptor
TMCS	Trimethylchlorosilane
UDP	Uridine 5'-diphospho-glucuronosyltransferase
VTG	Vitellogenin
WWTP	Waste water treatment plant
Wt1	Wilms tumor 1
3β-HSD	3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase
11β-hsd2	11β-hydroxysteroid dehydrogenase 2
11-KT	11-keto testosterone
17β-hsd12	17β-hydroxysteroid dehydrogenase 12

LIST OF PUBLICATIONS

Original research articles

1. **Harpreet Bhatia**, Anupama Kumar, Yukiko Ogino, Jun Du, Adrienne Gregg, John Chapman, Mike J. McLaughlin and Taisen Iguchi (2014) Effects of the commercial anti-androgen flutamide on the biomarkers of reproduction in male Murray rainbowfish (*Melanotaenia fluviatilis*). Environmental Toxicology and Chemistry 33(5): 1098 – 1107.
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1. **Harpreet Bhatia**, Anupama Kumar, John C. Chapman and Mike J. McLaughlin. Long-term exposures to di-n-butyl phthalate inhibit body growth and impair gonad development in juvenile rainbowfish (*Melanotaenia fluviatilis*). SETAC Asia/Pacific conference, Adelaide Sept 14-17, 2014.
2. **Harpreet Bhatia**, Anupama Kumar, Jun Du, John C. Chapman and Mike J. McLaughlin. Anti-androgen flutamide, alone and in combination with 17β-estradiol, impairs gonadal development in juvenile Murray rainbowfish (*Melanotaenia fluviatilis*). SETAC Asia/Pacific conference, Adelaide Sept 14-17, 2014.
3. **Harpreet Bhatia**, Kumar A, Yukiko O, Gregg A, Chapmann J, McLaughlin MJ and Iguchi T. Di-n-butyl phthalate causes antiandrogenic effects in male adult Murray rainbowfish (*Melanotaenia fluviatilis*). Society of environmental toxicology and chemistry conference. 3rd Australasia SETAC conference, University of Melbourne (Australia). October 1-3, 2013.
4. **Harpreet Bhatia**, Kumar A, Yukiko O, Du J, Gregg A, Chapmann J, McLaughlin MJ and Iguchi T. Adult male Murray rainbowfish (*Melanotaenia fluviatilis*) as a test model to assess antiandrogenic effects of flutamide in Australian riverine environment. 3rd Australasia SETAC conference, University of Melbourne (Australia). October 1-3, 2013.
5. **Harpreet Bhatia**, Anupama Kumar, Mike McLaughlin and John Chapmann (2012)

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6. **Harpreet Bhatia,** Kumar A and McLaughlin M. (2010) Endocrine effects of 17beta-trenbolone in fish – a feedlot contaminant. 3rd Australian Symposium on Ecological Risk Assessment and Management of Endocrine Disrupting Chemicals (EDCs), Pharmaceuticals and Personal Care Products (PPCPs) in the Australasian Environment, CSIRO Black Mountain, Canberra, ACT (Australia). Nov 10-11, 2010.

Presentations

1. **Harpreet Bhatia**, Anupama Kumar, John C. Chapman and Mike J. McLaughlin. Long-term exposures to di-n-butyl phthalate inhibit body growth and impair gonad development in juvenile rainbowfish (*Melanotaenia fluviatilis*). SETAC Asia/Pacific conference, Adelaide Sept 14-17, 2014.

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   3rd Society of Environmental Toxicology and Chemistry Conference. University of Melbourne. 1st – 3rd October 2013.

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   3rd Society of Environmental Toxicology and Chemistry Conference. University of Melbourne. 1st – 3rd October 2013.

POSTER PRESENTATION

5. **Harpreet Bhatia**, Anupama Kumar, John Chapman and Mike J. McLaughlin. Ovarian histopathology as a tool to evaluate endocrine disruption by di-n-butyl phthalate in adult female Murray rainbowfish (*Melanotaenia fluviatilis)*.

   2nd Society of Environmental Toxicology and Chemistry Conference. University of Queensland. 4th – 6th October 2012.

BEST POSTER PRESENTATION

6. **Harpreet Bhatia**. Gender benders in Australian waters. The University of Adelaide three-minute thesis competition. July 13, 2011

SECOND RUNNER-UP

7. **Harpreet Bhatia**, Anupama Kumar and Mike J. McLaughlin. Endocrine effects of 17β-trenbolone in fish: a feedlot effluent contaminant.

   3rd Australian Symposium on Ecological Risk Assessment and Management of Endocrine Disrupting Chemicals (EDCs), Pharmaceuticals and Personal Care Products (PPCPs) in the Australasian Environment, CSIRO Discovery Centre, Black Mountain, Canberra. 10th – 11th November 2010.

ABSTRACT

With the detection of anti-androgenicity in the effluents from the wastewater treatment plants (WWTPs), there is speculation that sexual disruption in fish is a multi-causal condition involving anti-androgens. Much of the research has focussed on deciphering the modes-of-action (MoAs) of (anti)estrogens and androgens. However, effects of androgen receptor (AR) antagonists have not been fully characterised and remain elusive in fish. The present study aimed to investigate the effects of the classic mammalian anti-androgen, flutamide and the emerging industrial pollutant, di-n-butyl phthalate (DnBP) on the biomarkers of reproduction in adult (male and female) and juvenile Murray rainbowfish (*Melanotaenia fluviatilis*). Flutamide is the "pure" anti-androgen designed to treat prostate cancer in men and polycystic ovarian syndrome in women. It has also been extensively used in toxicity testing in mammals. The *in vitro* anti-androgenic activity in the aquatic environment worldwide is measured in flutamide equivalents. Phthalates are a class of synthetic industrial chemicals commonly found in the aquatic environment worldwide. They have been recognised as anti-androgens in male mammals but little is known about their endocrine-disrupting effects in the native Australian fish species. Due to its detection in freshwater both in Australia and worldwide and considering its higher solubility in water (11 mg/L), it is important to investigate effects of DnBP on the reproductive fitness of native Australian fish species. Flutamide is not an environmental contaminant and has not been detected in freshwater. However, it is used as the reference chemical to quantify anti-androgenic activity in aquatic environment using *in vitro* assays. In addition, flutamide is also used as the model anti-androgen to investigate anti-androgenic effects in mammals.

Adult female and male Murray rainbowfish were exposed to biologically active concentrations (nominal 125 – 1000 µg/L) of flutamide for 7 days. In females, histological

investigation revealed marked atresia and absence of mature oocytes in the flutamide-treated fish at all concentrations investigated. Reduction in the sizes of the vitellogenic oocytes was found after treatment with 500 and 1000 µg/L flutamide. The plasma VTG and the activity of brain aromatase were reduced in fish treated with 500 and 1000 µg/L flutamide. Treatment with 500 and 1000 µ/L flutamide reduced the concentrations of 11-keto testosterone (11-KT) and 17β-estradiol (E2) in plasma. In males, qualitative assessment of the testes of the fish exposed to 125 – 1000 µg/L flutamide exhibited inhibition in transformation of spermatogonia to spermatozoa and increased testicular anomalies like multinucleated and pyknotic cells and interstitial fibrosis. VTG was induced in plasma after an exposure to 1000 µg/L of flutamide. The activity of brain aromatase declined after exposure to flutamide at all concentrations. Males exposed to 1000 µg/L of flutamide showed a down-regulation of the hepatic genes encoding androgen receptors α (ARα) and ARβ. The expression levels of the genes for the estrogen receptor α (ERα) were up-regulated and those of VTG were down-regulated after treatment with 250 – 1000 µg/L of flutamide.

Juvenile rainbowfish were exposed to the nominal concentrations 25 ng/L E2, 25 µg/L flutamide, 250 µg/L flutamide, 25 ng/L E2 + 25 µg/L flutamide and 25 ng/L E2 + 250 µg/L flutamide. Co-treatment with Flu high and E2 resulted in significant reductions in weights and lengths in males and condition factor in females. Inter-sex was noted in Flu high and E2+Flu high treated fish. The development of spermatocytes in the testes was inhibited by E2 and this effect was accentuated after co-treatment with flutamide. Exposures to E2 resulted in precocious oocyte development in the ovaries which was further up-regulated when fish were co-exposed to E2 and flutamide. The E2 levels decreased significantly in the head of both males and females after co-exposures to flutamide and E2. Flutamide and E2 alone increased the 11-KT levels in both sexes. However, E2+Flu low decreased 11-KT

levels in males and increased them in females. Flutamide (low and high) induced VTG protein in the tails of both sexes. In males, VTG was induced in the tail tissue after exposure to flutamide but not E2. No significant increase of flutamide on E2-induced VTG concentration was noted. We concluded that anti-androgens do not add to the effects of estrogens due to different modes of action. However, they induce similar effects which can cause additive inhibition/stimulation of the gonad development.

Sexually mature female and male Murray rainbowfish were exposed to sub-acute concentrations of 125 – 1000 µg/L DnBP for 7 days. The testes in 125 – 1000 µg/L DnBP-exposed fish were in the early spermatogenic stage with a higher proportion of spermatogonia. The sizes of spermatogonia, Type A and B spermatocytes and spermatids were significantly smaller relative to the controls after treatment with 125 – 1000 µg/L of DnBP. The sizes of the previtellogenic oocytes in the 250 – 1000 µg/L treated fish were higher than those in the corresponding control fish. The early vitellogenic oocytes in the 1000 µg/L treated fish were smaller relative to those in the unexposed fish. Histological changes like chorion folding, shrunken ooplasm, impaired yolk production, granulomatous inflammation and interstitial fibrosis were observed in the ovaries of the fish treated with DnBP at all concentrations. The plasma VTG was significantly lower in the female and higher in males exposed to 500 – 1000 µg/L DnBP. An induction in the expression levels of the genes encoding for ERα and β and choriogenin L, coupled with an amplified activity of aromatase in the brain for the 1000 µg/L of DnBP treatment suggested an estrogenic MoA of DnBP in male fish.

Juvenile fish were exposed to environmentally relevant concentrations (5, 15 and 50 µg/L) of DnBP for 90 days. The lowest observed effective concentration to significantly

affect the condition factor after 90 days was 5 µg/L. Histological investigation revealed complete feminisation of the gonad in fish exposed to 5 µg/L for 90 days and to 15 and 50 µg/L of DnBP at all sampling times. In addition, incidences of inter-sex gonads were noted in the 15 and 50 µg/L of DnBP treatments at the end of the exposure period. After 90 days of exposure to DnBP, the ovaries were regressed and immature. Testes, present only in fish exposed to 5 µg/L of DnBP for 30 or 60 days, were vacuolated and immature. There was a significant induction in E2 concentration in fish exposed to 5 µg/L of DnBP for 90 days and in 15 and 50 µg/L DnBP treatments at all sampling times. Long-term exposure to low concentration (5 µg/L for 90 days) had similar hormonal effects as short-term exposure to higher concentration of DnBP (50 µg/L for 30 days) in causing *in vivo* estrogenicity. Long-term continuous exposures to 5 µg/L of DnBP for up to 30 days did not have profound effects on body growth and gonadal differentiation of fish. However, 30 days of continuous exposures to 15 µg/L could interfere with the gonad development and to 50 µg/L could compromise the hormonal profile of juvenile fish.

The study, for the first time, reported the differential effects of two anti-androgens in male, female and juvenile Australian fish species. Using an integrated approach of histological, biochemical and molecular tools, the hypothetical models of effects and potential MoAs of flutamide and DnBP have been proposed. The data from the present study suggest that continuous exposures to biologically active concentrations of flutamide for 7 days can cause anti-androgenicity in male and defeminisation in female adult Murray rainbowfish. In addition, 35 day exposures to the anti-androgen, flutamide can induce feminisation in juvenile Murray rainbowfish and the effects of flutamide are cumulative in combination with E2. It was also concluded that continuous exposures to sub-acute concentrations of DnBP for 7 days can cause estrogenicity in male and anti-estrogenic effects

in female adult Murray rainbowfish. Treatments with environmentally relevant concentrations of DnBP for 90 days during sensitive phases of development in juvenile Murray rainbowfish adversely affect the fish growth and gonad development.

It is proposed that short-term exposures to high concentrations and long-term exposures to low concentrations of DnBP have similar reproductive endocrine effects. Australian water quality guidelines recommend DnBP concentrations should be < 9.9 µg/L for freshwater ecosystems (http://www.environment.gov.au/resource/australian-and-new-zealand-guidelines-fresh-and-marine-water-quality-volume-1-guidelines). Levels of DnBP found in freshwaters in Australia (47 ng/L) do not pose a threat to the reproductive fitness of Murray rainbowfish. However, there is a strong need to revise the water quality guidelines for DnBP in freshwater in Australia for future reference. It is also recommended to detect, identify and quantify individual anti-androgens in freshwater in Australia and worldwide. In addition, identification of the genes and testing molecular tools regulating gonadal differentiation in Murray rainbowfish are needed to assess the reversibility of the effects caused by environmental chemicals. It is important to investigate the endocrine disruption, if any, in the fish thriving in this aquatic environment in Australia using field caging studies. Similar studies have been reported in Europe and the US.

CHAPTER 1

REVIEW OF LITERATURE

1.1 Introduction

1.1.1 Endocrine disrupting chemicals in environment: concept

During the twentieth century, a large number of chemicals were developed for use in medicine, research, households, agriculture and industries. Although significant economic benefits are attributed to the use of these chemicals, including an increase in agricultural crop yields, better quality of finished product and various medical break-throughs, their widespread use has resulted in their release into the environment (Danzo, 1998) and possible bioaccumulation in wildlife. In recent years, compelling evidence has accumulated showing that minute concentrations of certain environmental chemicals can cause endocrine disruption in wildlife (Damstra et al., 2002; Hayes et al., 2002; Tyler et al., 1998). These chemicals have been termed as endocrine disrupting chemicals (EDCs). Endocrine disruption does not imply toxic or lethal responses; it refers to *in vivo* physiological or biochemical changes leading to adverse effects. An endocrine disruptor is "an exogenous substance or a mixture that alters the functions(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (Damstra et al., 2002).

1.1.2 Classes and sources of endocrine disrupting chemicals

A vast variety and number of chemicals (synthetic as well as natural) have been identified as EDCs because of their endocrine disrupting effects as demonstrated in *in vitro* and *in vivo* studies. Some EDCs share structural similarities with naturally occurring steroid hormones. Others are not structurally related and constitute a diverse group of chemicals

(Baker, 2001). The nature of the EDCs can broadly be classified into the following three categories –

a) *Natural steroids* – These are naturally synthesised compounds and are required for normal functioning of an organism. Endogenous steroidal hormones include the following –
    i. Estrogens, like 17β-estradiol (E2) and estrone (E1);
    ii. Androgens like testosterone (T) and
    iii. Phytoestrogens like genistein, β-sitosterol and coumestrol.
b) *Synthetic steroids* – These compounds are synthesised artificially by man to target the endocrine system to combat disease or improve resistance. These can further be classified into the following –
    i. Glucocorticoids – Prednisone, dexamethasone, triamcinolone, alcometasone.
    ii. Mineralo-corticoids – Fludrocortisones
    iii. Androgens – Apoptone, oxandrolone, oxabolone, nandrolone
    iv. Estrogens – Diethylstilbesterol, 17α-ethynylestradiol, diethylstilbestrol
    v. Progestins - danazol, norethindrone, medroxyprogesterone acetate, 17-hydroxyprogesterone caproate.
    vi. Anti-androgens – cyperoterone acetate, flutamide
    vii. Anti-estrogens - Tamoxifen
    viii. Anti-progestins - mifepristone, gestrinone
c) *Synthetic chemicals* – These chemicals are specifically designed for a variety of purposes like improving agricultural and dairy yield and protection from diseases. For example, DDT, organohalogens, organotins, alkylphenol ethoxylates, bisphenol A (BPA), phthalic acid esters (PAEs), 17β-trenbolone, DES, vinclozolin, dioxins,

PCBs and thyroid receptor antagonists (coumesterol and polybrominated dipheyl ethers) belong to this category.

The EDCs are released into the environment from a variety of sources which can be broadly classified into the following –

a) *Point sources* – These are the localised sources of EDC discharge. For example, the industrial discharges, effluents from the waste-water treatment plants (WWTPs), pulp and paper mill discharges and run-offs from cattle feedlots, dairies, piggeries etc.

b) *Diffuse sources* – These sources are spread over a certain region. For example, agricultural run-off, leaching from landfills etc.

Table 1 lists the classification and sources of some of the persistent EDCs.

Table1: Some natural and synthetic chemicals detected in environment.

Classification	Chemical	Source	References
Natural steroids	17β-estradiol, estriol, estrone, testosterone, progestins, medroxyprogesterone, androstenedione, 5α-dihydrotestosterone, 11-ketotestosterone, androsterone, progesterone	Livestock, dairy waste, human waste, wastewater treatment plant effluents, livestock/feedlot effluent	(Hanselman et al., 2003; Kim et al., 2014c; Kolodziej et al., 2003; Laurenson et al., 2014; Rao et al., 2014; Soto et al., 2004; Tashiro et al., 2003; Ying and Kookana 2002a; Ying et al., 2002b; Zhao et al., 2010)
Phytosteroids	β-sitosterol, zearalenone, coumesterol, lignans, isoflavanoids, genestein, stigmastanol, isopimaric acid, hydroxymatairesino, n-butylbenzene sulphonamide, dehydroabietic acid, betulin	Pulp and paper mill effluent	(Adebayo et al., 2014; Almstrup et al., 2002; Collins et al., 1997; Csaba, 2013; Gravina et al., 2013; Strauss et al., 1998)
Pharmaceuticals	tamoxifen, mestranol, diethylstilbestrol	Wastewater treatment plant effluents	(Chikae et al., 2004a; Laurenson et al., 2014; Ortiz-Zarragoitia et al., 2005; Van den Belt et al., 2004)
Herbicides	linuron, trifularin, atrazine, simazine	Agricultural run-off, wastewater treatment plant effluents	(Chen et al., 2014; de Lafontaine et al., 2014; Hayes et al., 2003; Koenen and Cavas et al., 2008; Rodriguez-Gonzalez et al., 2014; Tahara et al., 2014)
Insecticides	p,p'-DDE; o,p'-DDT; p,p'-DDT; o,p'-DDE, HPTE, methoxychlor, dieldrin, aldrin, chlordane, malathion, lindan/hexachlorcyclohexane, dicofol, permethrin, endosulphan, toxaphene, heptachlor, kepone, vinclozolin	Agricultural run-off, wastewater treatment plant effluents	(Davis et al., 2009; Glover et al., 2007; Harrahy et al., 2014; Helander et al., 2002; Jin et al., 2009; Luellen et al., 2006; Narita et al., 2014; Sanchez-Bayo and Hyne 2014; Uchida et al., 2010; Xia et al., 2009)
Phthalic acid esters	di-n-butyl phthalate, di-n-octyl phthalate,benzyl butyl phthalate, dimethyl phthalate, diisobutyl phthalate, diethyl phthalate, di-2-ethylhexylphthalate, diisononyl phthalate, diisodecyl phthalate	Leaching from plastics	(Kim et al., 2014a; Kim et al., 2014b; Takeuchi et al., 2005a; Xiaojing et al., 2013; Xue et al., 2014; Zacharewski et al., 1998; Zheng et al., 2014; Zhu et al., 2014; Zia et al., 2013)

Classification	Chemical	Source	Reference
Flame Retardants	pentabromodiphenylether (DE-71) and 2,2',4,4',6-penta BDE (DE-100)	Consumer products – fire resistant coatings	(Barco-Bonilla et al., 2014; de Jourdan et al., 2013; Liu et al., 2013; Martinez et al., 2013; Rawn et al., 2014; Vorkamp et al., 2014; Yu et al., 2010; Zhu et al., 2013)
Phenols	nonylphenol, bisphenol A, nonylphenol, pentaphenol, octylphenol, butylphenol	Wastewater treatment plant effluents, leaching from detergents	(Balch and Metcalfe 2006; Brown et al., 1999; Gray and Metcalf 1997; Gray et al., 1999; Kang et al., 2003; Ramakrishnan and Wayne 2008; Soverchia et al., 2005; Toomey et al., 1999; Zhang et al., 2014b)
Antifoulants	medetomidine, tamoxifen	Consumer products	(Chikae et al., 2004a; Legler et al., 2002; Mansour et al., 2012; Nebot et al., 2007; Zhao et al., 2011)
Organotins	tributyltin, triphenyltin	Consumer products	(Alzieu, 2000; Bones et al., 2006; Gooding et al., 2003; Hwang et al., 1999; St-Louis et al., 2000)
Organohalogens	dioxins; 2,4-dichlorophenol, furans, polychlorinated biphenyls, polychlorinated naphthalenes	Pulp and paper mill effluents	(Akerblom et al., 2000; Crews et al., 1995; Hashimoto et al., 2011; Heidler and Halder 2009; Matta et al., 1998; Mohnke and Buijten 1993; Smeets et al., 1999)
Polyaromatic hydrocarbons	benzopyrene, retene, benzanthracene, pyrene, 6-hydroxy chrysene, phenanthrene	Pulp and paper mill effluents	(Annammala et al., 2013; Billiard et al., 2004; Dasgupta et al., 2011; Estevez et al., 2012; Hodson et al., 2007; Kogila et al., 2013; Navas and Segner 2000; Oikari et al., 2002; Pilla et al., 2009; Sundt and Bjorkblom 2011)

Classification	Chemical	Source	References
Heavy Metals	arsenic, lead, mercury	Point sources	(Ben et al., 2014; Matta et al., 2001; Walker and MacAskill 2014; Wang et al., 2014; Wei et al., 2014; Zhang et al., 2014a)

1.2 Growing concern on endocrine disrupting compounds

There is now sufficient evidence to state that the populations of certain wild-life species are declining as a result of exposures to environmental chemicals. The frequency at which diseases are now seen in humans and in wildlife suggests exposures to environmental chemicals (WHO 2012). A 7-year, whole-lake study in Canada by Kidd and co-workers demonstrated that chronic exposures of fathead minnow (*Pimephales promelas*) to 5 – 6 ng/L EE2 could lead to extinction of the species by feminising the males through induction of vitellogenin (VTG) and adversely affecting oogenesis in females (Kidd et al., 2007). Tributyltin has been attributed to reduce the populations of snails (Titley-O'Neal et al., 2011). Modeling studies have predicted increases in concentrations of estrogens in South Australian and UK rivers in the future (Green et al., 2013) and declines in fish populations exposed to environmentally-relevant concentrations of estrogens over long periods (Grist et al., 2003). These impacts are difficult to assess in short-term laboratory studies. Robust laboratory studies are vital for our better understanding of the potential effects and modes of action of different environmental chemicals. Many laboratory and field studies conducted world-wide have investigated the effects of environmental chemicals identified as EDCs on general health, growth and reproduction in aquatic animals like fish. Since the publication of the book "Our Stolen Future" (Colborn, 1996), and due to the continuous popular media coverage of the issue, public concerns about the adverse effects of chemicals on reproductive systems of wildlife and human beings have grown immensely in Australia and overseas. Therefore, it is important that the scientific basis for managing this issue becomes established before alarmist reporting influences public opinion, so that rational decision making about the uses of a range of chemicals and wastes can occur. Scientists and managers need to be proactive to constructively contribute to the debate. In the following sections, some representative studies

highlighting the effects of environmental chemicals with (anti)estrogenic and (anti)androgenic mechanisms of action are collated.

1.2.1 Reproductive effects of estrogens in fish

One of the earliest studies demonstrated that the chemicals in the WWTP effluents were capable of inducing egg-precursor protein VTG in male rainbow trout (*Oncorhynchus mykiss*) (Purdom et al., 1994; Sumpter, 1995). Another major breakthrough was the detection of high incidence of intersexuality in wild roach (*Rutilus rutilus*) thriving in rivers receiving discharges with high estrogenic activity (Jobling et al., 1998). Disruptions in the development of secondary sexual features were also seen in hatchling snapping turtles (*Chelydra serpentina*) in St. Lawrence River basin containing polychlorinated biphenyls (de Solla et al., 2002). This was followed by researchers reporting a range of endocrine disrupting effects in wild fish populations living in receiving environment in the US, Europe, South Africa, Japan and Australia (Allen et al., 1999; Barnhoorn et al., 2004; Batty and Lim 1999; Bjerregaard et al., 2006; Campinho and Power 2013; Fensterheim, 2001; Folmar et al., 2001; Gercken and Sordyl 2002; Humble et al., 2014; Ibor et al., 2014; Kavanagh et al., 2004; Korner et al., 2000; Leusch et al., 2005; Montgomery et al., 2014; Vigano et al., 2001; Wang et al., 2013). The detection of estrogens in the range of 1 ng/L – 50 ng/L in the effluents in the UK, USA, Germany, Netherlands and Japan (Belfroid et al., 1999; Desbrow et al., 1998; Nasu et al., 2001; Stumpf et al., 1999), supported the induction in VTG levels in fish caged in close proximity to effluent discharges in rivers (Carlson and Williams 1999; Harries et al., 1996; Harries et al., 1997). A significant amount of research has focussed on the estrogens on invertebrates like waterfleas and snails (Baldwin et al., 1995; Oehlmann et al., 2000; Olmstead and LeBlanc 2000) and vertebrates like frogs and fish (Kloas et al., 1999; Lange et al., 2001; Metcalfe et al., 2001; Qin et al., 2011; Qin et al., 2007). Many laboratory studies

have reported feminising effects in fish after exposures to E2 (Brion et al., 2004; Falahatkar et al., 2014; Feswick et al., 2014; Humble et al., 2014; Tabata et al., 2001); alkylphenol ethoxylates (Gimeno et al., 1998; Gong et al., 2010; Hemmer et al., 2001; Jobling and Sumpter 1993; Routledge et al., 1998; Toomey et al., 1999; Vazquez et al., 2009; Yun et al., 2008); bisphenol (Duag et al., 2013; Kwak et al., 2001; Naderi et al., 2014; Woo et al., 2014); endosulfan (Bauer et al., 2013; Chow et al., 2013; Da Cuna et al., 2013; Dong et al., 2013; Tellez-Banuelos et al., 2013); and DDT (Leanos-Castaneda et al., 2002). Recent Australian studies observed that effluents from the WWTPs in New South Wales were estrogenic to mosquitofish (*Gambusia holbrooki*) (Rawson et al., 2008).

1.2.2 Reproductive effects of anti-estrogens in fish

Anti-estrogens block the utilisation of estrogens, thus, inhibiting the maintenance of female sex characteristics and development of ovaries. A 3400-fold increase in anti-estrogens in the sediments near the industrialized sites in comparison to those in the non-industrialised sites in USA was reported (Arcaro et al., 1999). Recent studies demonstrated that the anti-estrogen, malachite green suppressed the expression levels of growth hormone receptors (GHR) in black sea bream (Jiao and Cheng 2010). Further, this attenuation could not occur in the presence of ERs. This finding suggested the possible anti-estrogenic action of malachite green in fish. Another anti-estrogen, tamoxifen has been demonstrated to reduce VTG and E2 (Leaños-Castañeda et al., 2007) and masculinised the fry (Singh et al., 2012) in tilapia (*Oreochromis niloticus*). Williams and co-workers (Williams et al., 2007) conducted a full life-cycle (284 d) trial of tamoxifen citrate exposure to fathead minnows and observed significant effects on gonadal histology and VTG concentrations. Other chemicals like diethylstibestrol, genistein, methoxychlor and 4-tert-pentylphenol have also been

demonstrated to be anti-estrogenic in fish (Alam and Kawaskar, 1998; Blum et al., 2008; Chow et al., 2013; Martyniuk et al., 2011; Panter et al., 2002).

1.2.3 Reproductive effects of androgens in fish

Androgens are natural or synthetic chemicals that bind to the androgen receptors (ARs) in vertebrates leading to the stimulation, maintenance and development of male characteristics. Androgens are also the precursors of estrogens in females. Androgen disrupting chemicals mimic the physiological androgens by inducing androgen-receptor-mediated gene transcription. Although the majority of the research on the effects of EDCs on fish has focussed on the chemicals suspected of emulating natural estrogens, it is now abundantly clear that the reproductive physiology can be disrupted through a variety of mechanisms. Recent studies detected the presence of androgenicity in kraft mill influent and effluent samples in Canada (Werner et al., 2010). Parks and colleagues (Parks et al., 2001) observed that the female mosquito fish thriving in waters contaminated with pulp mill effluent have a 55% increase in the number of segments in the longest ray of the anal fin, a sign of masculinity. A body implant of 0.5 mg/kg of 17α-methyltestosterone (MT) resulted in the development of male-like cloaca and slender body in female grouper (*Epinephelus tauvinaI*) (Lee et al., 1995). Exposure of female fathead minnows to MT resulted in the appearance of tubercles and deeper body coloration (Pawlowski et al., 2004). At low concentrations (0.1 µg/L), MT was able to induce atresia and hamper oocyte development in female fathead minnow (Pawlowski et al., 2004) and the replacement of the ovary with a functional testis in female grouper (*Epinephelus tauvina*) (Lee et al., 1995). A reduction in the VTG levels was reported in eel (*Zoarces viviparus*) after treatment with MT and the effects were reversed after treatment with E2 (Korsgaard, 2006). Many recent studies have

demonstrated androgenic effects of MT in fish (Beaven and Muposhi 2012; Kefi et al., 2012; Marjani et al., 2009; Montajami, 2012; Rajakumari et al., 2009; Salah et al., 2012; Wahbi and Shalaby 2010). Adult female mosquitofish exposed for 28 d to 10 µg/L 17β-trenbolone developed gonopodium (Sone et al., 2005). 17β-trenbolone (1-10 µg/L) resulted in the maturation of spermatozoa in 4 wk old male mosquitofish, a feature normally not feasible until 90 days. The same dose caused the transformation of the ovaries into ovo-testis in females (Sone et al., 2005). Other studies have also reported toxico-dynamic effects in fathead minnows (Schultz et al., 2013); reduction in EE2-induced VTG levels in eelpout (*Zoarces viviparus*) (Velasco-Santamaria et al., 2013); adverse effects on ovarian development in Japanese medaka (*Oryzias latipes*) (Flynn et al., 2013); impairment of female sexual behaviour in sand gobies (*Pomatoschistus minutus*) (Saaristo et al., 2009); elongation of anal fin and reduction in the hepatic expression of VTG mRNA mosquitofish (*Gambusia holbrooki*) (Brockmeier et al., 2013).

1.2.4 Reproductive effects of anti-androgens in fish

Anti-androgens antagonise the effects of physiological androgens by inhibiting AR-mediated gene expression. Recent studies in UK concluded that the feminisation of fish downstream of WWTPs has a "multicausal etiology" and is apparently the result of not only the estrogens but also of the anti-androgens (Jobling et al., 2009c). On similar lines, another recent study proposed that a mixture of polyaromatic hydrocarbons and phthalates with anti-androgenic properties shielded the effect of androgens in the European rivers (Weiss et al., 2009). Anti-androgenicity of flutamide, vinclozolin and linuron have been demonstrated *in vivo* in three-spined sticklebacks (*Gasterosteus aculetaus*) (Katsiadaki Ioanna et al., 2007), medaka (Kiparissis et al., 2003; Makynen et al., 2000a) and guppies (*Poecilia reticulata*) (Baatrup and Jung 2001; Bayley et al., 2002). At concentrations > 500 µg/L, flutamide

induced VTG and E2 in male fathead minnow (Jenkins et al., 2004). A persistent metabolite of dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (p,p'-DDE) has been shown to inhibit the gonopodium development and suppress sperm count in guppies (Bayley et al., 2002). Expousre of zebrafish (*Danio rerio*) and euryhaline flounder (*Platichthys flesus*) to pentabromodiphenylether (DE-71) increased the expression of gonadal aromatse (Kuiper et al., 2008). It was recently observed that AR antagonist cyproterone acetate inhibited the appearance of secondary sexual characters like the nuptial tubercles in fathead minnows (Ankley et al., 2010). A dose of 1 mg/fish of cyproterone acetate to catfish (*Clarias batrachus*) inhibited spermatogenesis, regressed the seminal vesicles and reduced the testosterone levels in plasma (Singh and Joy 1998). Exposure of 1 week old medaka larvae to flutamide induced ova-testis and disrupted spermatogenesis in males (Leon et al., 2007). The presence of estrogens and anti-androgens was recently detected in 30 UK rivers (Jobling et al., 2009b). Supporting it was the detection of anti-androgenic compounds in the bile of fish exposed to the WWTP effluents in UK (Hill et al., 2010).

Although *in vitro* studies have detected anti-androgenic activity in aquatic environment world-wide, the presence and fate of anti-androgenic chemicals in the Australian riverine environment has not been evaluated. Currently, we have limited understanding of the impacts of anti-androgens on the aquatic wildlife in Australia. International studies have shown significant impacts of anti-androgens on reproduction in fathead minnows, zebrafish and stickleback. It is, therefore, crucial that an understanding of the effects of anti-androgens is gained in Australian fish species. Since *in vitro* studies have demonstrated that anti-androgens can cause phenotypic feminisation, it is important to investigate their effects in robust laboratory experiments. In the following sections, the effects of the "pure" anti-androgen, flutamide and the emerging industrial contaminant, di-n-butyl phthalate with

potential anti-androgenicity on the biomarkers of reproduction in mammals and overseas fish species are discussed.

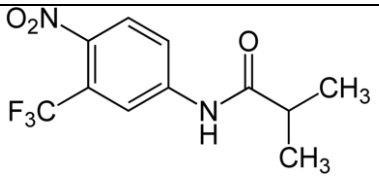
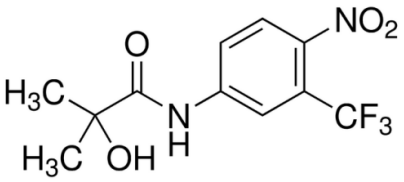
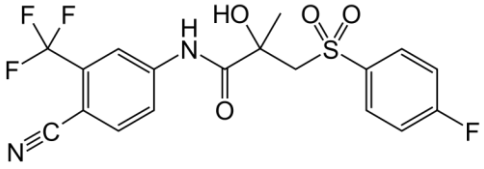
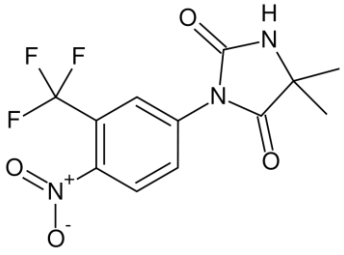
1.3 Flutamide

1.3.1 Flutamide – nature and uses

Prostate cancer accounts for up to 29% of the cancer cases and is the leading cause of cancer-related deaths in the USA (Siegel et al., 2011). It is the second most common form of cancer, after lung cancer, in Australia. It is predicted that the number of men diagnosed with prostate cancer could increase to 267,000 by 2017 in Australia (www.prostate.org.au). The ARs play important roles in the progression of prostate cancer in males. Upon binding to the androgens, the ARs undergo conformational change and bind to and activate the androgen-responsive genes. Thus, castrations due to prostate cancer can be prevented by using hormone therapy. Hormone therapy aims to either reduce androgen availability or block ARs. However, prostate cancer can develop via ARs even after androgen suppression (Feldman and Feldman 2001). Consequently, blocking the ARs is a key to control the progression of prostate cancer. Steroidal (cyperoterone acetate, cyperoterone, oxendolone and spironolactone) chemicals and non-steroidal (flutamide, enzalutamide, bicalutamide, nilutamide and cyanolutamide) chemicals have been synthesised to block the binding of androgens to ARs. The steroidal anti-androgens have lower affinity relative to the non-steroidal ones to bind to the ARs. In addition, the lack of tissue specificity and the potential side-effects due to cross-reactivity with other receptors (like cyperoterone acetate suppresses gonadotropin production and spironolactone is mineralocorticoid receptor antagonist) makes the steroidal anti-androgens less suited for use as AR antagonists. The “pure antiandrogen” flutamide (2-methyl-N-[4-nitro-3-(tri-fluoromethyl) phenyl]propanamide) was the first non-

steroidal anti-androgen synthesised and has been demonstrated to reduce mortality from advanced prostate cancer effectively (Delaere and Vanthillo 1991; Labrie et al., 1995). Flutamide has been demonstrated to compete with testosterone to bind to ARs (Ankley et al., 2004; Simard et al., 1986). Inside the body, flutamide is rapidly absorbed in the gastro-intestinal tract and undergoes metabolism into 2-hydroxyflutamide and hydrolysis product 3-trifluoromethyl-4-nitroaniline. 2-hydroxyflutamide has a higher affinity than flutamide to bind to the ARs (Wakeling et al., 1981). 3-trifluoromethyl-4-nitroaniline has been related to the flutamide-induced hepatotoxicity (Fau et al., 1994). Table 2 depicts the chemical names and structures of the common non-steroidal anti-androgens.

Table 2: Chemical structures of some of the commonly used non-steroidal anti-androgens

Anti-androgen	Chemical name	Chemical structure
Flutamide	2-methyl-*N*-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide	
2-Hydroxy flutamide	2-Hydroxy-2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide	
Bicalutamide	*N*-[4-cyano-3-(trifluoromethyl)phenyl]-3-[(4-fluorophenyl)sulfonyl]-2-hydroxy-2-methylpropanamide	
Nilutamide	5,5-dimethyl-3-[4-nitro-3-(trifluoromethyl)phenyl] imidazolidine-2,4-dione	

1.3.2 Reproductive effects of flutamide

1.3.2.1 Mammalian studies

Due to the strong anti-androgenicity and potentially no cross-reactivity with other receptors, flutamide is used in toxicity testing in mammals. Several mammalian studies have reported demasculinisation in males after exposure to flutamide. The anti-androgenic effects included reduction in sperm counts and motility, penile length, circulating levels of testosterone, ano-genital distance and the Sertoli cell numbers.

Flutamide administration during critical stages of development can interfere with the androgen-regulated processes and cause irreversible changes in mammals (Mikkila et al., 2006). *In utero* exposure to up to 30 mg/kg body weight (BW) flutamide for 5 days resulted in the development of vaginal pouches in male pups (Goto et al., 2004). Interestingly, feminisation of external genitalia and cryptorchidism were observed after *in utero* exposure to flutamide. However, these reproductive effects were not apparent when rats were administered flutamide in postnatal period (Kassim et al., 1997). The reproductive behaviour (like mounting a female) was not affected upon flutamide administration during neonatal period. However, prenatal exposure continued till neonatal phase lowered adult mounting behaviour (Brand and Slob 1991).

Impairments in sperm development and motility after *in utero* or neonatal exposures to flutamide have been well-documented (Perobelli et al., 2012; Vo et al., 2009a). It has been suggested that reduction in the levels of testosterone in epididymis as well as the reduced action of androgens result in accelerated sperm transit. This causes reductions in sperm motility and fertilising potential (Perobelli et al., 2012). Reduction in the number of sperms in epididymis have also been reported after an embryonic exposure to flutamide (Anway et al., 2008). Treatment with flutamide can cause a decrease in the expression of facilitative glucose transporter (GLUT-5) protein in spermatozoa. This protein is essential for storage of sperms in female genital tract (Roy and Krishna 2013). Flutamide inhibited sperm maturation in pubertal pigs (Maschio et al., 2010). Disruption of cell-to-cell contacts have been reported in foetal gonads in pigs (Hejmej et al., 2012; Knapczyk-Stwora et al., 2013; Kopera et al., 2011; Kopera et al., 2010).

Decrease in testosterone concentrations accompanied by increase expression of aromatase mRNA have been reported in pigs after administration of flutamide (Kotula-Balak et al., 2012). Ultrastructural evidence suggests that flutamide can cause an increase in the frequency of organelles involved in steroid hormone synthesis in the Leydig cells (Maschio et al., 2010). On similar lines, a 41% reduction in testosterone concentrations in rats after administration of 25 mg/kg BW flutamide for 2 weeks has been attributed to the changes in the Leydig cells (Perobelli et al., 2012). Leydig cell hyperplasia/hypertrophy has been reported in rats after *in utero*, perinatal or adult exposure to flutamide (Mikkila et al., 2006; Mylchreest et al., 1999). It has been suggested that flutamide exposure during neonatal period has a long-term effect on the Leydig cell development leading to androgen-estrogen imbalance (Kotula-Balak et al., 2012). *In utero* exposure to flutamide has been demonstrated to downregulate the expression of the mRNA for connexin-43 (gap junction protein) in pre-antral follicles in female pig (Durlej et al., 2012) (Table 3).

Table 3: Some of the past literature on the effects of flutamide on the biomarkers of reproduction in mammals

Animal	Route	Dose[a]	Effects	References
Pig	GD90 (for 7 d) GD108	50	Delayed folliculogenesis, decline in E-cadherin expression crucial for follicular development, increase in the expression levels of TNFα mRNA consistent with increased apoptosis	(Moody et al., 2013)
	GD90 (for 7d)	50	Decrease in gap junction-associated connexion and adheren junction-associated beta-catenin in fetal gonads leading to disrupted testiculat functions and impaired follicle formation	(Knapczyk-Stwora et al., 2013)
	GD20-GD28	50	Increase in Leydig cell size, reduction in testosterone, increase in aromatase mRNA expression,	(Kotula-Balak et al., 2012)
	GD20, GD80 IP	50	Decrease in expression of ZO-1, N-cadherin and beta-catenin	(Hejmej et al., 2012)
	PND2	50	Spermatogenic arrest, germ cell detachment, germ cell epoptosis, , Leydig cell hyperplasia,	(Kopera et al., 2011)
	30d old for 10 days	10	Spermatogonial maturation and differentiation, detachment of primary spermatocytes, increased volume of Leydig cells, accumulation of myelin bodies in Leydig and Sertoli cells	(Maschio et al., 2010)
	GD-20-28, GD80-88		Reduction in Sertoli cell number, multinucleated germ cells, delayed folliculogenesis, decreased expression of Cx43 in gonads	(Kopera et al., 2010)
Rat	Oral for 8 wk	20	Increase in serum levels of gonadotropins and androgens	(Chandolia et al., 1991)
	GD19 GD22 PND 1-5	15	Reduction in testes and prostate weights, percentage of normal sperms, sperm concentration and testosterone level in male offspring Implantation losses and reduced offspring size from mating of normal females with treated males	(Leonelli et al., 2011)
	GD13.5 – GD21.5 PND 1-15	100	Inhibition of penis growth, increase in AGD	(van den Driesche et al., 2011)
	PND21-35	1-50	Hyperplasia of Leydig cells, decrease in the expression of the genes for StAR, Cyp11A1, HSDβ3,	(Vo et al., 2009b)
	GD11-21	1-50	Increase in testosterone and LH levels, reduction in seminiferous tubules	(Vo et al., 2009a)
Bat	Adult 7d	100	Dose-dependent decrease in the expression of GLUT-5 protein in testes	(Roy and Krishna 2013)

[a]Represents dose at mg flutamide per kg body weight; GD: Gestation day; IP: Intraperitoneal; PND: Post-natal day

1.3.2 Fish studies

Some research in aquatic toxicology has focussed on the elucidation of the effects of anti-androgens in fish. However, most anti-androgens are not "pure" and tend to bind the ERs at certain concentrations. Fungicide and mammalian anti-androgen, vinclozolin and its metabolites 2-[(3,5-dichlorophenyl)-carbamoyl]oxy-2-methyl-3-butenoic acid (M1) and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) failed to bind to the ARs in the brain and ovary in fathead minnows, suggesting that these chemicals might not be anti-androgenic in fish (Makynen et al., 2000b). Another category of mammalian anti-androgens, phthalates bind to the ERs at higher concentrations (Asai et al., 2000). The complete role of AR in fish is not well researched (Borg, 1994). Hence, the effects of anti-androgens in reproductively active fish remain elusive. There is a need to establish the effects and possible modes of action (MoA) of anti-androgens in fish using strong AR antagonist like flutamide. Some overseas studies have investigated the effects of flutamide in fathead minnow and three-spined stickleback. The results from these studies are in corroboration with the anti-androgenic effects of flutamide observed in rats. Although flutamide has not been detected in the aquatic environment worldwide, it could be an emerging contaminant in future due to the vast usage of this pharmaceutical. In addition, the detection of anti-androgenic activity of 100 – 1000 flutamide equivalents per litre (FEQ/L) in aquatic environment makes flutamide an ideal anti-androgen for toxicity testing in fish laboratory studies.

Exposure of sexually mature fathead minnows to nominal concentration (651 µg/L) flutamide for 21 days resulted in reductions in embryo hatch and fecundity (Jensen et al., 2004). In addition, the ovaries of the flutamide-treated fish contained higher proportion of atretic oocytes and testes developed necrosis. Induction of E2 and VTG was measured in plasma of the flutamide-exposed males (Jensen et al., 2004). Flutamide has been reported to

effectively block the 17β-trenbolone-induced nuptial tubercle development in female fathead minnow (Ankley et al., 2004). Trenbolone is a strong AR agonist chemical. This *in vivo* study confirmed that the anti-androgenic actions of flutamide are mediated via ARs. In another study, male stickleback administered 100 – 1000 µg/L flutamide for 21 days was demasculinised. It produced significantly lower amount of spiggin, built fewer nests and showed reduction in the frequency of the zig-zag movements (sexual courtship behaviour) towards the females (Sebire et al., 2008). In one study, Japanese medaka was exposed to 125 - 1000 µg/L flutamide for a short duration (juvenile for 28 days and adults for 21 days). It was noted that exposure during early life-stage severely delayed the papillary-process development, while no significant effect was observed in adult fish (Nakamura et al., 2013). The development of papillary processes in medaka is regulated by androgens. This study is another example of the anti-androgenic effects of flutamide on the development of secondary sexual features in fish (Table 4).

Flutamide has been demonstrated to produce feminising effects like induction of VTG in males. It has been suggested that flutamide competes with androgens to bind to the ARs. This displaces testosterone from ARs. Testosterone levels can increase in the plasma leading to its conversion to E2. E2 rapidly binds to the ERs and initiates the cascade of events resulting in feminisation (like VTG production). It has also been demonstrated that exposure to 320 µg/L flutamide or 10 ng/L EE2 feminises the male fathead minnows. However, the gene profile of the exposed fish suggest different MoAs of the two chemicals. For instance, EE2 up-regulated the gene expression of ERα and flutamide of ERβ and ERγ in males. Flutamide up-regulated the expression of the genes involved in androgen biosynthesis while EE2 down-regulated these genes (Filby et al., 2007). It is, therefore, of interest to study the effects of flutamide in combination with estrogens.

Table 4: Endocrine and reproductive effects of exposure to flutamide in fish from past research

Fish	Stage	Sex	Concentration	Route	Time	Effects	References
Medaka (*Oryzias latipes*)	Embryo	Male	320 µg/L	Water	96 h followed by 90 d post exposure	induction of testis ova	(Leon et al., 2007)
	Adult	Male	101 – 1560 µg/L	Water	21 d	induction of intersex gonads reduced fecundity and fertility	(Kang et al., 2006)
Guppy (*Poecilia reticulata*)	Adult	Male	0.1 – 100 µg/g	Food	30 d	reduction in ejaculated sperms, smaller testes, disruption in male courtship behaviour	(Baatrup and Jung 2001)
	Juvenile	Male	0.1 – 100 µg/g	Food	Until sexual maturity	inhibited growth disruption in male sexual behaviour, reduced gonopodium	(Bayley et al., 2002)
	Adult	Male	1 – 100 µg/mg	Food	30 d	Decrease in the number of spermatogenic cysts	(Kinnberg and Toft 2003)
Three-spined stickleback (*Gasterosteus aculeatus*)	Quiescent adult	Male	100 – 1000 µg/L	Water	21 d	low spiggin level, decrease in the number of nests built, disruption of courtship behaviour	(Sebire et al., 2008)
	UD (Wild fish)	Male	250 µg/L	Water	21 d	Inhibition of 17α-methyltestosterone (100 ng/L) induced spiggin production	(Katsiadaki et al., 2006)
	NM	Female	250 µg/L	Water	21 d	Inhibition of DHT-induced spiggin production	(Pottinger et al., 2013)

Fish	Stage	Sex	Concentration	Route	Time	Effects	References
Fathead minnow (*Pimephales promelas*)	Adult	Male	50 – 500 µg/L	Water	21 d	reduced fecundity, low embryo hatch, decrease in mature oocytes, increased oocyte atresia in progeny	(Jensen et al., 2004)
		Females				elevated 17β-estradiol, increased vitellogenin	
		Females	400 µg/L	Water	14 d	Blocking of 17β-trenbolone induced nuptial tubercle production.	(Ankley et al., 2004)
	50 dph Juvenile	ND	33 µg/L	Water	50 d	Reduction in transcription factors involved in testicular development (*dmrt1*, *sox9a*, *wt1, Ad4BP/SF-1*), testicular differentiation (*nr2c1*), steroid hormone synthesis (*StAR, 11β-hsd2, 17β-hsd12, P450c17*)	(Rajakumar et al., 2012)
	Adult	Females	100 µg/L	Water	7 d	Increase in brain aromatase activity	(Andersen et al., 2003)
	Adult	Females	700 µg/L	Water	14 d	Decrease in E2, oocyte atresia	
Asian catfish (*Clarias batrachus*)		Males		Water		Decrease in T and 11-KT	(Bottero et al., 2005)
Zebrafish (*Danio rerio*)	Juvenile	2 yo	1250 mg/kg BW	Food	2 wk	Decrease in E2, VTG, P450 content	(Shilling and William 2000)

Dmrt1: doublesex/mab-3 related transcription factor; Sox9a: sex determining region Y-box 9; wt1: Wilms tumor 1; Ad4BP/SF-1: adrenal 4 binding protein/steroidogenic factor 1; Nrc1: HR-associated Cell death 1; StAR: Steroid acute regulatory protein; 11β-hsd2: 11β-hydroxysteroid dehydrogenase 2; 17β-hsd12: 17β-hydroxysteroid dehydrogenase 12; P450c17: Cytochrome P450, family 17, subfamily A, polypeptide 1; UD: Undifferentiated; NM: Not mentioned; dph: days before hatch; BW: Body weight

1.4. Phthalic acid esters

1.4.1 Phthalic acid esters – nature, uses and prevalence

After the detection of anti-androgenic activities in the aquatic environment worldwide, there has been an increasing concern to identify the anti-androgens in freshwater and elucidate their potential effects and MoAs in fish. This has shifted the focus of environmental scientists from estrogens to anti-androgens. One of the classes of emerging anti-androgenic contaminants is phthalic acid esters (PAEs) or phthalates. Phthalates are an important class of industrial chemicals, commonly known as plasticisers in media. Phthalates were first synthesised in the early 20th century and have since been used widely in the production of printed inks, cosmetics, gloves, toys, pesticides etc. They are mixed with polyvinyl chloride to provide flexibility to the plastics by lowering their glass transition temperature (Graham, 1973). Since phthalates are not chemically bound to the plastics, they leach out over time (Autian, 1973).

Chemically, phthalates are alkyl or aryl esters of 1,2-benzene dicarboxylic acid and are commercially manufactured by condensation of alcohols with phthalic anhydride. The structure of phthalates consists of a benzene ring with two carboxyl groups attached at the ortho position. In addition, there are linear or branched alkyl chains ranging from 1 to 10 carbon atoms (Table 5). The lengths of their chains impart varying physical and chemical properties and hence, uses and biological effects to the phthalates. Phthalates with up to four carbon atoms are used in pharmaceuticals and non-vinyl commercial products, while C8 to C10 PAEs are used as additives to impart flexibility to the plastics. Water solubility of PAEs decreases with increase in the carbon content of the side chains and determines its bioaccumulation potential, aquatic availability and biodegradation (Staples et al., 1997a). For example, two of the most common phthalates, diethylhexyl phthalate (DEHP) and di-n-butyl

phthalate (DnBP) have molecular weights of 390.6 and 278.4 g/mol, respectively. Their water solubilities are 2.5 µg/L and 9900 µg/L respectively. Vapour pressure determines the release PAEs and their metabolites into the atmosphere. Some of the physical properties of the PAEs are listed in Table 6.

Table 5: Chemical structures of phthalic acid and the two most common environmental pollutants, di-butyl phthalate and diethylhexyl phthalate

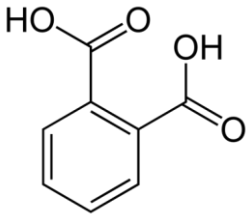
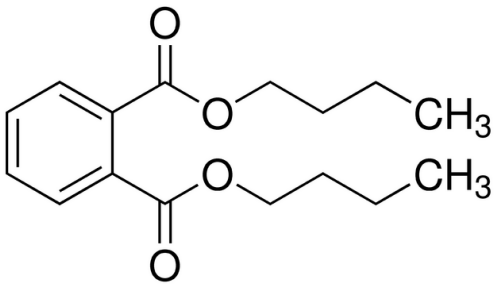
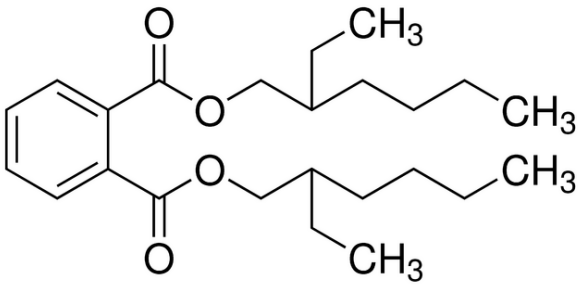
Common name	**Chemical name**	**Chemical formula**
Phthalic acid	Benzene-1,2-dicarboxylic acid	
DBP	1,2-Benzenedicarboxylic acid dibutyl ester	
DEHP	Phthalic acid bis(2-ethylhexyl ester)	

Table 6: Physical properties of various types of phthalates.

Phthalate acid ester	**Acronym**	**CAS**	**MW (g/mol)**	**Water solubility (µg/L)**	**Vapour pressure (Pa)**
Dimethyl phthalate	DMP	13-11-3	194.2	5,220,000	0.263
Diethyl phthalate	DEP	84-66-2	222.4	591,000	0.0648
Diallyl phthalate	DAP	131-17-9	246.3	156,000	0.0271
Dipropyl phthalate	DPrP	131-16-8	250.3	77,000	0.0175
Dibutyl phthalate	DBP	84-74-2	278.4	9,900	0.00473
Butylbenzyl phthalate	BBP	85-68-7	312.4	3,800	0.00249
Diphenyl phthalate	DPhP	84-62-8	318.3	3,093*	0.00000136*
Dicyclohexyl phthalate	DCHP	84-61-7	330.4	4,000*	0.00000087*
Di-n-hexyl phthalate	DnHP	84-75-3	334.5	159	0.000345
Di-2-ethylhexyl phthalate	DEHP	117-81-7	390.6	2.5	0.0000252
Dioctyl phthalate	DOP	117-84-0	390.6	2.5	0.0000252
Diisononyl phthalate	DiNP	28553-12-0	418.6	0.31	0.00000681
Diisodecyl phthalate	DiDP	26761-40-0	446.7	0.038	0.00000184
Monomethyl phthalate	MMP	4376-18-5	180.2	5,958,000*	0.0318*
Monoethyl phthalate	MEP	2306-33-4	194.2	1,212,000*	0.0098*
Mono-n-butyl phthalate	MnBP	131-70-4	222.2	893,000	0.00516*
Monobenzyl phthalate	MBzP	2528-16-7	256.3	51,700	0.0000546*
Mono-iso-butyl phthalate	MiBP	30833-53-5	222.2	977,000	0.0031*
Mono-2-ethylhexyl phthalate	MEHP	4376-20-9	278.4	111,000	0.000109*
Mono-C6/8/10 phthalate	$MC_{6/8/10}P$			131,000	
Mono-iso-nonyl phthalate	MiNP	59056-28-1	292.4	56,000	0.0000464*
Mono-C8/10 phthalate	$MC_{8/10}P$			46,000	
Monodecyl phthalate	MDP	24539-60-4	306.4	130*	0.0000131*

*Represents estimated figures.

Data from (Cousins et al., 2003) and (Staples et al., 2011).

Industrial chemicals like phthalates have a high incidence of entering the aquatic environment due to their widespread production, incorrect disposal or accidental spillage during the manufacture or industrial processing and also due to leaching from the consumer products (Leblanc, 1980). Industrial wastewater in Netherlands has been reported to contain up to 150 µg/L DEHP and 21 µg/L DnBP (Vethaak et al., 2005). Up to 1.5 mg/L diethyl phthalate (DEP) has been detected in the influents of WWTPs in South Africa (Olujimi et al., 2012). The EDCs are not removed fully from the waters in the WWTPs. For instance, WWTPs in China were reported to remove 18 – 46% of E1, EE2 and bisphenol A (BPA) from the influents (Qing et al., 2013). Phthalates have been detected in the STP effluents and

suspended particulate matter in the UK (Fatoki and Vernon., 1990), Germany (Fromme et al., 2002) and Netherland (Vethaak et al., 2005). During the last decade, reclaimed water from China was found to contain 1.8 µg/L DEHP (Huang et al., 1999). Effluents from the WWTPs or STPs are the major contributors of these congeners in the freshwater ecosystems (Olujimi et al., 2012). High concentrations of PAEs have been detected in the freshwaters in Nigeria (1.5 mg/L DBP) (Fatoki et al., 1993), China (6 µg/L DEHP) (Shi et al., 2012), Malaysia (64.3 µg/L DEHP) (Tan 1995) and Netherlands (5 µg/L DEHP) (Vethaak et al., 2005). Concentration of DBP up to 33.5 µg/L DBP were found in the freshwaters in UK in 1984 (Fatoki and Vernon 1990). Less than two decades later, 75.6 µg/L DBP was detected (Fatoki and Noma 2001), suggesting increasing pollution of freshwaters by PAEs. Table 7 lists the levels of phthalates detected in the aquatic environment worldwide.

Table 7: Reported concentrations of various phthalates in aquatic environment worldwide

Source	Place	Year	PAE	Levels	Reference
Influent	The Netherlands	1999	DMP	390 – 6200 ng/L	(Vethaak et al., 2005)
			DEP	4.1 – 44, 000 ng/L	
			DBP	< 0.38 – 51 µg/L	
			DPP	< 1 – 6, 700 ng/L	
			BBP	560 – 4, 900 ng/L	
			DMPP	1.9 – 15, 000 ng/L	
			DCHP	< 11 – 210 ng/L	
			DEHP	< 13 – 100 µg/L	
			DOP	260 – 2400 ng/L	
	Australia	2007	DEP	1080 ng/L	(Tan et al., 2007)
			DBP	201 ng/L	
			BBP	134 ng/L	
			DEHP	716 ng/L	
STP effluent	The Netherlands	1999	DMP	< 3 – 320 ng/L	(Vethaak et al., 2005)
			DEP	< 300 – 930 ng/L	
			DBP	< 420 – 840 ng/L	
			DPP	< 1 – 22 ng/L	
			BBP	< 70 – 290 ng/L	
	China	2003	DBP	< LOD – 8.1 ng/L	(Wang et al., 2005)
			DEHP	1 – 23.8 µg/L	
			DiBP	< LOD – 14.9 ng/L	
	USA	2001	DMP	2.36 µg/L	(Loraine et al., 2006)
			DEP	2.10 µg/L	
			DBP	3.71 µg/L	
			BBP	0.651 µg/L	
			DEHP	4.49 – 20.7 µg/L	
	Australia	2007	DEP	4.9 ng/L	(Tan et al., 2007)
			DBP	34.3 ng/L	
			BBP	75.7 ng/L	
			DEHP	589 ng/L	
Industrial effluent	The Netherlands	1999	DMPP	< 1 – 20 µg/L	
			DCHP	< 2 – 20 ng/L	(Vethaak et al., 2005)
			DEHP	< 470 – 2400 ng/L	
			DOP	< 2 – 19 ng/L	
			DMP	< 2 – 1300 ng/L	
			DEP	< 350 – 5200 ng/L	
			DBP	< 0.69 – 210 µg/L	
			DPP	< 4 – 460 ng/L	
			BBP	< 170 – 1300 ng/L	

Source	Place	Year	PAE	Levels	Reference
Rainwater	The Netherlands	1999	DMPP	< 0.73 – 400 µg/L	(Vethaak et al., 2005)
			DCHP	< 5 – 160, 000 ng/L	
			DEHP	1 – 1500 µg/L	
			DOP	12 – 2800 ng/L	
			DMP	8 – 18 ng/L	
			DEP	240 – 430 ng/L	
			DBP	280 – 880 ng/L	
			DPP	< 50 ng/L	
			BBP	140 – 260 ng/L	
Freshwater	The Netherlands	1999	DMPP	380 – 530 ng/L	(Vethaak et al., 2005)
			DCHP	< 8 ng/L	
			DEHP	690 – 1700 ng/L	
			DOP	38 – 250 ng/L	
			DMP	< 4.5 – 190 ng/L	
			DEP	< 70 – 2300 ng/L	
			DBP	< 66 – 3100 ng/L	
			DPP	< 1.9 – 8 ng/L	
			BBP	< 10 – 1800 ng/L	
	UK	1984	DMPP	50 – 2400 ng/L	(Fatoki and Vernon 1990)
			DCHP	< 3 – 60 ng/L	
			DEHP	< 900 – 5000 ng/L	
			DOP	< 2 – 78 ng/L	
			DBP	21.5 µg/L	
	Nigeria	1990	DBP	< LOD – 1472 mg/L	(Fatoki et al., 1993)
			DEP	< LOD – 538 mg/L	
			DMP	< LOD – 462 mg/L	
		2002	DMP	< LOD – 21.03 mg/L	(Ogunfowokan et al., 2006)
			DEP	< LOD – 6.55 mg/L	
			DPP	< LOD – 30.26 mg/L	
			DBP	< LOD – 53.81 mg/L	
			DEHP	17.82 – 80.53 mg/L	
			DOP	< LOD	
			DiNP	< LOD	
	Malaysia	1992	DBP	0.8 – 3.2 µg/L	(Tan 1995)
			DEHP	3.1 – 64.3 µg/L	
	South Africa	NM	DMP	< LOD – 350 µg/L	(Fatoki and Noma, 2002)
			DEP	< LOD – 398.3 µg/L	
			DBP	< LOD – 102.8 µg/L	
			DEHP	2.1 – 2306.8 µg/L	
	Spain	2001	DBP	< LOD – 2.36 µg/L	
			DEP	< LOD – 16.2 µg/L	
	France	2005	DEP	71 – 181 ng/L	(Dargnat et al., 2009)
			DBP	67 – 181 ng/L	
			DEHP	160 – 970 ng/L	

DBP: Dibutyl phthalate, DEHP: Diethyl hexyl phthalate; DEP: Diethyl phthalate; DOP: Diocytl phthalate; DiNP: Di-iso-nonyl phthalate; DOP: Dioctyl phthalate; DMP: Dimethyl phthalate; DCHP: Dicyclohexyl phthalate; BBP: Butyl benzyl phthalate; DPP: Dipropyl phthalate; DMP: Dimethyl phthalate

Once in the environment, phthalates are rapidly adsorbed on the surface of sediments. This, combined with their low water solubility and quick biodegradation into phthalic acid and alcohol, and subsequently to carbon dioxide and water (Blount et al., 2000; Perez et al., 1976; Scholz, 2003; Staples et al., 1997a), limits their availability to the aquatic organisms (Staples et al., 1997b). However, their ubiquitous usage and subsequent continual release into the rivers pose a threat to the reproductive health and sexual behaviour of aquatic wildlife. Phthalates can enter the fish body via water through gill respiration, skin, ingestion of suspended particles and consumption of other organisms containing phthalate residues (Barnhoorn et al., 2004). Phthalates were detected in the muscle of bream (*Abramis brama*) (1.5 µg/g w/w DEHP) and flounder (*Platichthys flesus*) (0.144 µg/g w/w DEHP) in the freshwaters of the Netherlands (Vethaak et al., 2005). The bioaccumulation of phthalates has been confirmed in the laboratory environment as well. DEP residues have been known to bioaccumulate in gills, muscle, liver, brain and testis of carp (*Cyprinus carpio*) after 28 days of exposure to 20 µg/L (Barse et al., 2007). Phthalates can easily pass into the blood stream from the skin and gills due to their lipophilic nature.

1.4.2 Reproductive effects of phthalic acid esters

Human exposure to phthalates is generally through the consumer products like cosmetics, pharmaceuticals, medical devices, food packaging, cleaning materials etc (Schettler, 2006). The detection of phthalates or their metabolites in the urine has been used

as an indication of phthalate exposure to humans (Frederiksen et al., 2011; Koch et al., 2003). Methyl hexyl phthalate (MEHP), the metabolite of DEHP and the oxidative metabolites of MEHP, mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) have been detected in the urine of humans in the US (Barr et al., 2003). In another study, MEHP (2.7 µg/L), monobutyl phthalate (MBP) (21.2 µg/L), monobenzyl phthalate (MBzP) (41 µg/L) and monoethyl phthalate (MEP) (305 µg/L) were detected in human urine in the US (Blount et al., 2000). Up to 1.4 mg/L DEHP was detected in Finnish human breast milk and the same study directly related these values to impairments in Leydig cell development (Main et al., 2006). The urine of men working with polyvinyl chloride was found to contain 100 times higher levels of MEHP than that of the unexposed men (Pan et al., 2006). Increasing risk of testicular dysgenesis syndrome (including cryptorchidism, hypospadias, testicular cancer and reduction in sperm quality) in men is being linked to the exposure to phthalates (Hu et al., 2009). This has necessitated a thorough investigation of the reproductive effects of phthalates using rat as the vertebrate model.

1.4.2.1 Mammalian studies

It has now been established that exposure to phthalates during critical phases, gestational day (GD) 12 – GD21, can cause severe and irreversible impairments to the reproductive development and sexual behaviour in mammals (Table 8). For example, in a multigenerational study, sexually mature male and female rats administered up to 1% DnBP in diet for 14 wk did not show significant changes in reproduction. However, the F1 generation when matured had reduced rates of fertility, mating and pregnancy (Wine et al., 1997). *In utero* exposure of rats to phthalates has also been reported to cause cryptorchidism (Hu et al., 2009; Imajima et al., 1997; McKinnell et al., 2005) which is comparable to testicular dysgenesis syndrome in humans (Fisher et al., 2003). Studies have suggested that

this could be due to reduced concentrations of androgens. Insulin like3 (INSL3) is produced by the Leydig cells in the testes of mammals and it regulates the descent of the testes. The production of insulin like peptide-3 (INSL3) is upregulated by testosterone (Anand-Ivell et al., 2009). *In vitro* studies have reported that MEHP suppressed the action of testosterone on INSL3 mRNA in Leydig cells of rats (Lague and Tremblay 2008). Similar results have been reported after *in vitro* culture of foetal testis of rat with 10 µM MEHP (Chauvigne et al., 2011).

It has been well documented that administration of phthalates can suppress *in vivo* androgen-regulated molecular and physiological processes, leading to anti-androgenicity in males. Recent *in vitro* studies have confirmed the anti-androgenic potency of phthalates in mammals. Rat foetal testis xenografts exposed for 4 days to 500 mg DBP/kg BW/d showed a significant reduction in the weight of the seminal vesicles and circulating levels of testosterone (Mitchell et al., 2012). Foetal rat testis cultured for 3 days with up to 10 µM MEHP resulted in reduced testosterone, 5α-dihydrotestosterone and androstenedione levels (Chauvigne et al., 2011). Many mechanisms have been proposed for the reduction in androgen levels caused after exposure to phthalates. Phthalates have been reported to downregulate the mRNA of the enzymes involved in steroidogenesis like cytochrome P450 17A1 (CYP17A1), cholesterol side chain cleavage enzyme (P450scc), scavenger receptor class B type 1 (SR-B1) and StAR in the Leydig cells in the testes leading to decrease in androgen production (Mitchell et al., 2012). Proliferation of the Leydig cells observed after treatment with phthalates could be to compensate the reduced androgen-production (Mahood et al., 2005). Another study reported an increase in anti-mullerian hormone (AMH) after exposure to DnBP in rats (Moody et al., 2013). Downregulation of AMH is critical for the maturation of Leydig cells and production of testosterone (Mendis-Handagama and

Ariyaratne 2001; Trbovich et al., 2001). In addition, DBP suppressed the expression of the activin A by the upregulation of inhibin production (Moody et al., 2013). Activin A has been implicated in Sertoli cell proliferation and androgen production.

Phthalates have also been reported to result in inhibition of germ cell development in the testes in mammals. *In vivo* and *in vitro* studies have demonstrated that exposures of mice to MEHP decreased the expression of occludin in the tight junctions between the Sertoli cells and laminin-gamma-3 and beta-1-integrin between the Sertoli cells and germ cells (Yao et al., 2010). Exposure to MEHP destroyed the vimentin filaments in the Sertoli cells (Richburg and Boekelheide 1996). MEHP has also been reported to increase the levels of FASL, a transmembrane protein belonging to tumour necrosis factor (TNF), leading to apoptosis of germ cells (Yao et al., 2010). *In vitro* exposure of 7 – 12 gestational week human foetuses to 10^{-4} M MEHP for 72 h resulted in a decrease in the proportion of germ cells in the testis (Muczynski et al., 2012).

Although most studies on the biological effects of phthalates have been done using male members of mammalian species, their potential adverse effects on the female reproductive system cannot be overlooked. A few studies have also investigated the effects of phthalates on the endocrine function in female rats. *In utero* exposures of female mice to up to 1000 mg MEHP/kg BW/d for two days has been reported to cause anti-estrogenicity as evidenced by decreased aromatase expression, reduction in the circulating levels of E2 and late onset of sexual maturity (Moyer and Nixon 2012). 96-h exposures to 1 – 100 µg/mL DEHP and 0.1 – 10 µg/mL MEHP inhibited the growth of the antral follicles and reduced E2 production and the expression levels of mRNA of aromatase (Moyer and Nixon 2012). Administration of 15 mg/kg BW phthalates from GD 6 to PND 22 resulted in delays in

vaginal opening (Grande et al., 2007). DEHP has been implicated in reducing the circulating levels of E2 (Davis et al., 1994), pre-implantation losses (Davis et al., 1994) and increased risk of abortions (Ema et al., 2000).

Several *in vitro* studies have demonstrated that phthalates like DBP can bind the estrogen receptors and initiate estrogenic responses (Andersen et al., 1999; Harris et al., 1997; Jobling et al., 1995a; Soto and Sonnenschein 1995; Zacharewski et al., 1998). Not much research has focussed on elucidating the estrogenic effects of phthalates. Given the fact that phthalates can bind to the ERs *in vitro*, it is vital to investigate their *in vivo* interaction with the ERs. Since ERα and ERβ play different physiological roles, the effect of phthalates on the expression levels of their genes can provide a clear picture of the mechanisms underlying the feminising effects. In addition, *in vitro* yeast assays have reported that BBzP can exhibit anti-androgenicity, in addition to estrogenicity (Sohoni and Sumpter 1998). Hence, the receptor-based MoA of phthalates remains elusive. Phthalates with 3 – 6 carbon atoms have been reported to bind and induce the expression of human ERα and this induction was inhibited by the anti-estrogen, tamoxifen (Takeuchi et al., 2005b). However, the estrogenic potency of phthalates is very low. Ecological risk analysis has proposed that the environmental estrogens have the highest estrogenic activity (10^{-1} to 10^{3} ng-E2/L) followed by phenolic compounds (10^{-3} to 10 ng-E2/L) while that of PAEs was negligible (Sun et al., 2013). DnBP and its metabolite, monobutyl phthalate (MBP) have been reported to exhibit AR antagonist activity at lower concentrations (EC50 values of 1.05×10^{-6} M and 1.22×10^{-7} M, respectively) and AR agonist activity at higher concentrations (EC50 6.17×10^{-6} M and 1.13×10^{-5} M, respectively) in an *in vitro* study using human breast cancer cells (Shen et al., 2009). It has been hypothesised that at higher concentrations some potent antiandrogens induce a receptor conformation able to bind to the ARs, thus, causing AR induction (Wong

and Gill 1995). PAEs exhibit agonist or antagonist activities via steroid hormone receptors (Takeuchi et al., 2005a) and these activities are regulated by EDC-induced changes in the conformation of the receptors, concentration of the EDCs, presence of co-factors in the cells and the presence or absence competing natural ligands (Miyamoto et al., 1998; Shen et al., 2009; Wong and Gill 1995). Dibutyl benzyl phthalate (DBBzP) (1 µM) was demonstrated to displace up to 75% of the tritiated E2 from the ERs in rainbow trout (Matthews et al., 2000). The binding of DBP to ERα in humans is disrupted by a potent anti-estrogen, tamoxifen, thereby, suggesting that PAEs can assert their effects through the ERs (Nakai et al., 1999; Takeuchi et al., 2005a). However, early *in vitro* work reported that the binding of PAEs to the ERs did not show parallelism to the binding of E2 to the ERs (Jobling et al., 1995b). This indicates that PAEs could have additional mechanisms of action.

Table 8: Some past research on the reproductive effects of phthalates in mammals

Animal	Phthalate	Route	Dose[a]	Duration (d)	Effects	References
Mice	DnBP	Oral	500	4 do – 10 do	Delayed spermatogenesis, impaired sertoli cell maturation, elevated inhibin, decrease in serum testosterone and testicular androgen, reduced AGD	(Moody et al., 2013)
Mice	DEHP	GST	500	GD7 – GD14	Reduced sperm count and motility in F1 to F4 offspring, disruption in testicular germ cell organisation and spermatogonial stem cell function in transgenerational manner	(Doyle et al., 2013)
Rats	DnBP	GST/ LAC	500	GD 14.5 – PN D6	Reduced AGD at PND10, smaller testes at PND 24, high levels of INSL3 (foetal hormone) and its mRNA at PND24 (puberty)	(Ivell et al., 2013)
Rats	DnBP DEHP	GST	66 750	GD 8 – GD 14	Reduction in ovarian and uterine weights in F1 offspring, reduction in seminal vesicle and epididymal weights azoospermic and atretic seminiferous tubules, apoptotic spermatogenic cells in F3 offspring, Epimutations consisting of 197 differential DNA methylation regions in gene promoter regions of sperms in F3 offspring	(Manikkam et al., 2013)
Rats	BBP	GST	25	GD 14 – GD 21	Metabolites perturbed in brain and testes in male pups and in uterus in female pups	(Banerjee et al., 2012)
Mice	MEHP	Oral	1000	12 h	Detachment of spermatocytes and spermatids, increase in the lumen size, decrease in the proportion of spermatids	(Yao et al., 2010)
Rats	MEHP	GST	1000	GD 17 – GD 19	Delayed oestrous, increase in serum FSH and E2, mammary gland hyperplasia, reduction in aromatase, StAR and LHCGR mRNA in F1 offspring	(Moyer and Nixon 2012)

Animal	Phthalate	Route	Dose[a]	Duration (d)	Effects	References
Marmosets	DEHP	Oral	2500	3 mo – 65 mo	Increase in ovarian and uterine weights, enlarged corpus luteum	(Tomonari et al., 2006)
Rats	DnBP	Oral	500	3 mo (3 days exposure)	Increase in proliferation and delay in maturation of Leydig cells, increase in the expression of the transcripts for INSL3, LHR, Cyp17a1 and Cyp11a1	(Heng et al., 2012)

[a]Represents mg phthalate per kilogram body weight per day

GD – Gestational day, EMB – Embryonic, PND – Postnatal day, GST – Gestational, LAC – Lactational, AGD – Anogenital distance, INSL3 – Insulin-like 3, do – day old

1.4.2.2 Fish studies

With the widespread detection of phthalates in the aquatic environment worldwide and the demonstration of the reproductive effects in mammals and humans by phthalates, it is likely that aquatic animals like fish are prone to endocrine disruption by phthalates. Fish can absorb EDCs through gills during respiration, dermal exposure and ingestion of food particles (Streit, 1998). During gill respiration, the EDCs do not undergo metabolism in the liver and are readily transferred to the blood stream and then taken to the target tissues.

Liver is the main reserve of lipids and a source of energy in non-fatty fish. Hepatosomatic index (HSI), a measure of the relative weight of the liver, has often been used as a measure of the glycogen-reserve in the body. Exposures to sub-acute concentrations of PAEs have been known to cause an increase in HSI. Male zebrafish (*Danio rerio*) exposed for 10 days to a high concentration of DEHP (5000 mg/kg) were found to have higher HSI in comparison to the controls (Uren-Webster et al., 2010). Similar findings have been reported in carp (*Cyprinus carpio*) reared for 28 days in water containing 20 mg/L DEP (Barse et al., 2007).

Histopathological evidence suggests that the peroxisomes in the liver proliferate after treatment of fish with PAEs (Ortiz-Zarragoitia et al., 2005; Ortiz-Zarragoitia et al., 2006) which could explain the increase in HSI in the PAE-treated fish. Peroxisome proliferation also augments the peroxisome proliferator-activated receptors (PPARs), nuclear hormone family receptors that bind to and are activated by lipophilic molecules like steroid hormones. Recently, *in vitro* treatment of zebrafish hepatocytes with up to 500 nM DEHP was reported to upregulate the transcripts for PPARα, PPARβ and PPARγ in both males as well as females (Maradonna et al., 2013). It has been proposed that di-alkyl phthalate esters readily

metabolise into their corresponding mono-alkyl esters *in vivo* (Williams et al., 1975). These mono-alkyl ester metabolites bind to and activate PPARs more rapidly than the parent diester (Uren-Webster et al., 2010). Supporting this hypothesis is a recent study wherein the MEHP was demonstrated to adversely affect PPARα in the liver of goldfish (*Carrasius auratus*), consequently, altering lipid metabolism (Jordan et al., 2012). The activated PPARs then undergo heterodimerisation with retinoid X receptor. This nuclear receptor complex then binds to the peroxisome proliferator-response elements (PPREs) in the promoter region of the target gene to induce hepatic toxicity and modulate biochemical pathways involved in β-oxidation of fatty acids, amino acid metabolism, glucose homeostasis, hepato-carcinogenesis etc and differentiation of adipocytes (Cho et al., 2012; Matsusue et al., 2004; Wong et al., 2002). Peroxisome proliferation was observed in chick embryos treated with 100 µM DEHP for 48 h (Arias, 2012).

In spawning periods, considerable lipid reserves of the body are utilised in the process of oocyte maturation and incorporation of VTG, an egg-yolk precursor protein, into the oocyte. Since up to 50% of a molecule of VTG can be composed of phospholipids, disruption in lipid metabolism can infer interference in the process of vitellogenesis (Jordan et al., 2012). However, Uren-Webster and co-workers did not find any significant change in the transcript profiles of PPAR-regulated genes (*acyl-coenzyme A oxidase 1 [acox1], hydratase/3-hydroxyacyl coenzyme A dehydrogenase [ehhadh]* and *lipoprotein lipase [lpl]*) after exposure of zebrafish to DEHP (Uren-Webster et al., 2010), thereby, suggesting more mechanisms for phthalate-induced hepatic toxicity in fish.

A few studies have reported adverse effects of PAEs on the normal process of oocyte growth and development. Treatment of juvenile Japanese medaka with 10 µg/L of

DEHP for 3 months resulted in the failure of the oocytes to mature (Kim et al., 2002). The proportion of the ovulated eggs was significantly reduced in zebrafish (*Danio rerio*) after treatment with up to 40 µg/L DEHP for 3 weeks (Carnevali et al., 2010). An *in vitro* study demonstrated that the zebrafish oocytes treated with DEHP and BBP failed to undergo germinal vesicle breakdown (GVBD) (Tokumoto et al., 2005).

Several mechanisms of the effects of phthalates in oocyte development in fish have been suggested. Prostaglandins are a class of signalling molecules known to play important roles in the process of oocyte maturation. The reduction in prostaglandin-endoperoxide synthase 2 (ptgs2) levels in zebrafish after treatment with 0.2 to 40 µg/L DEHP suggest that the adverse effects of PAEs on oocyte development could be through prostaglandins (Tokumoto et al., 2005).

The transformation of the oocyte from perinucleolar to the successive stages of development is accompanied by the growth of the oocyte and accumulation of cortical alveoli. By the late vitellogenic stage, the contents of the cortical alveoli are released in the periphery of the oocyte forming an acellular layer, chorion, composed of choriogenin. Choriogenin, in turn, is composed of glycoproteins synthesised from hepatic precursor proteins – choriogenin-H (ChgH) and choriogenin-L (ChgL) (Sugiyama et al., 1999). Chorion prevents polyspermy by forming ε-(γ-glutamyl) lysine crosslinks catalysed by transglutamase (Ha and Iuchi 1997). Formation of chorion is, thus, vital for the transformation of previtellogenic oocytes to vitellogenic stage. Phthalic acid esters have been reported to induce the hepatic *vtg* transcript in the hepatocytes *in vivo* (Barse et al., 2007; Christiansen et al., 2000; Uren-Webster et al., 2010) and *in vitro* (Maradonna et al., 2013) in fish. Genes for the ERs have been implicated in the process of vitellogenesis in largemouth bass

(*Micropterus salmoides*) and rainbow trout (Leaños-Castañeda and van der Klaak 2007; Sabo-Attwood et al., 2004). A significant increase in the levels of hepatic c*hg-L* transcript was also observed in the fish exposed to 1000 µg/L of DnBP. The transcript and protein for choriogenin L – c*hg-L* and Chg-L, respectively are the more sensitive biomarkers of exposure to environmental estrogens than VTG and *ER*s (Arukwe et al., 2000; Celius et al., 2000; Fujita et al., 2004). Induction of *ChgL* transcript and VTG protein after exposure to DnBP indicate an estrogenic mode of action of PAEs in male fish.

Adverse effects after administration of phthalates have also been reported in the testes of mammals and fish. Administration of 100 mg/kg DBP during critical phases of development resulted in the induction of ERα and ERβ levels in the testes along with Leydig cell hyperplasia after 9 weeks of post-natal development (Wakui et al., 2013). Zebrafish treated with 500 mg/kg DEHP for 10 days showed a reduction in the proportion of spermatocytes (Uren-Webster et al., 2010). Atlantic salmon (*Salmo salar*) fed a diet containing up to1500 mg/kg DEHP for 4 weeks immediately after the yolk-sac absorption were found to contain 3% intersex condition in the later stages of the life-cycle (Norman et al., 2006), thereby, suggesting that exposure to PAEs during sensitive or critical periods of the life-cycle can induce gonadal abnormalities. Treatment of medaka with up to 10 µg/L DEHP for 2 - 3 weeks post-hatch caused adverse effects on the process of their natural maturation (Chikae et al., 2004b; Shioda and Wakabayashi 2000). Exposure of fathead minnows to nominal concentration 100 µg/L BBP did not affect fecundity. However, the spawning frequency was reduced (Harries et al., 2000). A recent study investigated the effect of exposure to up to 2 g/kg DnBP on sexual differentiation in European pikeperch (*Sander lucioperca*). Testicular development was delayed with increased frequency of intersex condition (Jarmolowicz et al., 2013).

Attempts have also been made to assess the hormone concentrations in fish after phthalate treatment. In one study, carp treated with high concentrations (up to 20.5 mg/L) DEHP for 48 h had high circulating levels of testosterone and low levels of E2 (Han et al., 2009). Incubation of testicular microsomes with 100 μM DBP and tritiated androstenedione resulted in 41% decline in the production of DHT. Incubation of ovarian microsomes with 1 mM DBP led to an increase in the production of 17 α-20α-dihydroxy-4-pregnen-3-one and 17α,20β-dihydroxy-4-pregnen-3-one (Thibaut and Porte 2004). It has been suggested that a decrease in the abundance of cytochrome P4503A4 (CYP3A4) in liver could be responsible for decline in hormone concentrations after exposure to phthalates at environmentally relevant concentrations (Crago and Klaper 2012). Table 9 lists some of the studies reporting biologically active concentrations of phthalates. Table 10 lists some of the studies reporting the biologically active concentrations of different phthalates.

Table 9: No observed effect concentrations, lowest observed effect concentrations and lethal concentration of selected phthalates in different classes of organisms

Phthalate	Organism	NOEC (mg/L)	LOEC (mg/L)	LC-50 (mg/L)	References
DBP	*Daphnia magna*			10.35 (24 h)	(Adams et al., 1995; Huang et al., 1999; Wilson et al., 1978)
	Bluegill (*Lepomis macrochirus*)			0.48 (96 h)	
	Rainbow trout (*Oncorhynchus mykiss*)			1.2 – 6.47 (96 h)	
	Red killifish			4.3 (96 h)	
	Fathead minnow			0.61 – 3.95 (96 h)	
	Zebrafish (*Danio rerio*)			2.2 (96 h)	
	Yellow perch			0.35 (96 h)	
	Green algae (*Scenedesmus obliquus*)			0.21 (96 h)	
	Red tide algae (*Gymnodinium breve*)			0.2 (96 h)	
DEP	Common carp (*Cyprinus carpio*)			48 (96 h)	(Adams et al., 1995; Barse et al., 2007; Buccafusco et al., 1981; Ghorpade et al., 2002; Leblanc, 1980; Wilson et al., 1978)
	Mrigal (*Cirrhina mrigala*)			50 (72 h)	
	Bluegill (*Lepomis macrochirus*)			16.7 – 110 (96 h)	
	Rainbow trout (*Oncorhynchus mykiss*)			12 (96 h)	
	Fathead minnow			16.8 – 31.8 (96 h)	
	Red tide algae (*Gymnodinium breve*)			6.1 (96 h)	
	Water flea (*Daphnia magna*)			52 (48 h)	
DMP	Bluegill (*Lepomis macrochirus*)			50 (96 h)	(Adams et al., 1995; Jaworska et al., 1995; Leblanc, 1980; Wilson et al., 1978; Yan et al., 1995)
	Rainbow trout (*Oncorhynchus mykiss*)			56 (96 h)	
	Fathead minnow			39 (96 h)	
	Chlorella pyrenoidosa			313 (96 h)	
	Tetrahymena pyriformis	100 (48 h)	200 (48 h)	537 (96 h)	
	Red tide algae *(Gymnodinium breve)*			96 (96 h)	
	Water flea (*Daphnia magna)*				

Phthalate	Organism	NOEC (mg/L)	LOEC (mg/L)	LC-50 (mg/L)	References
BBP	Bluegil (*Lepomis macrochirus*)			43 (96 h)	(Adams et al., 1995; Buccafusco et al., 1981; Leblanc, 1980)
	Rainbow trout (*Oncorhynchus mykiss*)			0.82 – 3.3 (96 h)	
	Fathead minnow			0.78 – 5.3 (96 h)	
	Water flea (*Daphnia magna*)			92 (48 h)	
DEHP	Mussel (*Mytilus edulis*)	0.042 (28 d)			(Buccafusco et al., 1981; Leblanc, 1980)
	Brook trout (*Salvelinus fontinalis*)	0.052 (150 d)			
	Water flea(*Daphnia magna*)			11 (48 h)	
	Bluegill (*Lepomis macrochirus*)			>770 (96 h)	
DPP	Red tide algae (*Gymnodinium breve*)			2.4 (96 h)	(Wilson et al., 1978)

NOEC: No-observed effect concentration; LOEC: Lowest-observed effect concentration; LC: Lethal concentration

Table 10: Some of the past laboratory studies reporting reproductive, biochemical, histological, molecular and/or morphological effects of selected phthalates in fish

Fish	Sex	Stage	Age	Anti-androgen	Route	Concentration	Duration	Effects	References
Bagrid catfish (*Pseudobagrus fulvidraco*)	UD	UD	9 mph	DBP/DEHP	D	1000 mg/kg	8 wk	Brain AChE increased	(Jee et al., 2009)
Three-spined stickleback (*Gasterosteus aculetaus*)	M	AD	NM	DBP	W	35 ug/L	22 d	Increase in T, 11-KT levels and decrease in spiggin production	(Aoki et al., 2011)
Common carp (*Cyprinus carpio*)	M/F	AD	NM	DEHP	W	25 mg/L	48 h	Decrease in cholesterol and AA	(Han et al., 2009)
	M	AD	NM	DEP	W	0.1 – 20 mg/L	28 d	Increase in ALP; VTG and decrease in AcP, AST, GSI	(Barse et al., 2007)
Mrigal (*Cirrhina mrigala*)	NM	AD	NM	DEP	W	25 mg/L	72 h	Increase in liver ACP, ALP, ALT; muscle SDH and decrease in brain AChE	(Ghorpade et al., 2002)
Olive flounder (*Paralichthys olivaceus*)	NM	AD	NM	DEP	IP	900 mg/kg	3 d	Increase in liver GR, GPx, renal LPO, Serum ALP, LDH, AST, ALT and decrease in liver GSH, GST, CAT, AST, ALT	(Kang et al., 2010)
Atlantic salmon (*Salmo salar*)	UD	EMB	NM	DEHP	D	400-1500 mg/kg	4 w	Intersex	(Norman et al., 2006)

Fish	Sex	Stage	Age	Anti-androgen	Route	Concentration	Duration	Effects	References
Zebrafish (*Danio rerio*)	F	UD	6 m	DEHP	W	0.02 – 40 ug/L	3 w	Increase in ovarian BMP-15, ptgs2, VTG and decrease in LHR, mPRβ	(Carnevali et al., 2010)
	M	AD	NM	DEHP	IP	5000 mg/kg	10 d	Increase in HSI, hepatic *vtg*, *acox1*, *ehhadhIand* decrease in Fertilisation success, spermatozoa	(Uren-Webster et al., 2010)
Japanese medaka (*Oryzias latipes*)	UD	LV	2 wk	DINP/DIDP	D	20 ug/g	81 dph	Increase in testosterone hydroxylase activity in males	(Patyna et al., 2006)
	UD	LV		DEHP	W	0.01 – 10 ug/L	> 4 w	Increased mortality, Hatching time and decreased BW	(Chikae et al., 2004b)
	M	AD	10-15 m	DEHP	W	0.1 - 1 umol/L	2 w	No affect on eggs and hatchings	(Shioda and Wakabayashi 2000)
	UD	LV	PH	DEHP	W	0.01 – 10 ug/L	3 wk	Reduction in GSI in males, adverse impacts on the normal maturation process	(Chikae et al., 2004c)
Rainbow trout (*Oncorhynchus mykiss*)	M	AD	NM	DBP	IP	50 mg/kg	3 d	No affect on UDP-glucuronyltransferase, GST, EROD	(Cravedi and Perdu-Durand 2002)
Guppy (*Poecilia reticulata*)	UD	LV	< 1 wk	DEHP	W	0.1 - 10 ug/L	91 d	Decrease in body length, body weight	(Zanotelli et al., 2010)

Fish	Sex	Stage	Age	Anti-androgen	Route	Concentration	Duration	Effects	References
Fathead minnow (*Pimephales promelas*)	M	AD	NM	DEHP	W	12 ug/L	28 d	Increase in FSHβ, StAR, CYP11A, 17βHSDH and decrease in E2, 3βHSDH, CYP17, CYP19A1, PPARα	(Crago and Klaper 2012)
Zebrafish (*Danio rerio*)	UD	EMB	4 hpf	DBP/DEP	W	5 – 500 ug/L	Till 96 hpf	Increase in reactive oxygen species and lipid peroxidation; increase in the activities of superoxide dismutase, catalase and glutathione peroxidise; upregulation of the transcripts for interferon γ, interleukin-1β, myxovirus resistance, tumor necrosis factor α, CC-chemokine, lysozyme and complement factor C3B.	(Xu et al., 2013)
Goldfish (*Carassius auratus*)	UD	NM	NM	DEHP	W	2050 ng/L	10 d	Disruption of lipid metabolism in liver and and amino acid metabolism in gonads	(Jordan et al., 2012)
Sterlet (*Acipenser ruthensus*)	*In vitro* study with spermatozoa					10 – 50 µg/L		Dose dependent decrease in sperm motility and increase in DNA fragmentation and oxidative stress in sperms	(Gazo et al., 2013)

AChE: Acetyl choline esterase; 11-KT: 11-Ketotestosterone; T: Testosterone; AA: Aromatase: ALP: Alkaline phosphatase; ACP: Acid phosphatase; AST: Aspartate transaminase; GST: Glutathione S-transferase; GSH: Glutathione; CAT: Catalase; LDH: Lactate dehydrogenase; LPO: Lipid peroxidase; GR: Glutathione reductase; GPx: Glutathione peroxidase; BMP: Bone morphogenetic protein; LHR: Luteinising hormone receptor; PR: Progesterone receptor; EROD: Ethoxyresorufin-o-deethylase; FSH: Follicle stimulating hormone; UDP: Uridine 5'-diphospho-glucuronosyltransferase; F: Female; M: Male; UD: Undifferentiated; NM: Not mentioned; AD: Adult; LV: Larval; D: Diet; W: Water; IP: Intra-peritoneal

1.5 Gaps in Aquatic Ecotoxicology research

Based on the literature review, some gaps in the aquatic ecotoxicology research from Australian perspective were identified.

1.5.1 Limited research on the effects via androgen receptors of environmental contaminants in freshwater fish.

The EDCs can interfere with the hormonal system through estrogen, androgen, thyroid or retinoid receptors (Hotchkiss et al., 2008). Since the detection of widespread sexual disruption male roach thriving in water bodies receiving estrogenic effluents from the STPs (Jobling et al., 1998), most research in endocrine disruption in aquatic wildlife has predominantly focussed on the effects associated with estrogenic substances present in the WWTP effluents. Not much research has focused on the addressing the effects and MoA via ARs of environmental contaminants. Using *in vitro* screens, anti-androgenic activity has been detected in the aquatic environment in China (Ma et al., 2013; Zhao et al., 2011). Recently, statistical modelling suggested that anti-androgenic activity in the effluents from WWTPs could be contributors to the intersex condition seen in wild fish (Jobling et al., 2009a). Hence, there is a need to conduct laboratory studies on the effects of anti-androgens on the biomarkers of reproduction in fish.

1.5.2 Lack of substantial research on the effects of anti-androgens on fish during critical stages of their development.

The reproductive endocrine system is regulated by the hypothalamo-pitutary-gonadal (HPG) axis. The proteins and hormones released from the hypothalamus and pitutary act on the gonads and vice versa. The proteins and hormones in fish can be structurally different from those in mammals. However, they are chemically analogous and perform the same

functions. For example, the main androgen in mammals is testosterone and 11-keto testosterone, a derivative of testosterone is the predominant androgen in male fish. Consideration towards mammalian studies was important in designing the experiments because the endocrine system in vertebrates is highly conserved. This evolutionary conservation suggests that the chemicals demonstrated to cause endocrine disruption in mammals can adversely affect the biomarkers of reproduction in fish.

1.5.3 Paucity of studies highlighting differential effects of anti-androgens in male and female fish.

Most studies on anti-androgens have focussed on male fish. However, it has been well documented that androgens are essential for the growth of oocytes in fish (Endo et al., 2011; Kazeto et al., 2011; Kortner et al., 2009a,b; Matsubara et al., 2003; Sudo et al., 2012). Secondly, *in vivo* production of androgens during ovulation can induce aggression in females, thereby, enhancing their choice of males (Tricas et al., 2000). Third, female fish are equally likely to absorb anti-androgenic EDCs from aquatic environment. This necessitates the investigation of the potential effects via ARs of contaminants in female fish thriving in freshwater environment.

1.5.4 Need to elucidate the effects of anti-androgens in combination with estrogens.

With the detection of anti-androgenic activity in the aquatic environment worldwide, the roles of anti-androgens in causing sexual disruption in fish are being investigated. Further, statistical modelling data suggest that the anti-androgens, along with estrogens, could be responsible for the widespread endocrine disruption of the fish in English rivers (Jobling et al., 2009a). In addition, *in vitro* reports have confirmed that anti-androgens can behave as

estrogens (Sohoni and Sumpter 1998). This has necessitated conducting robust laboratory exposures investigating the effects of anti-androgens in conjugation with estrogens.

1.5.5 Validation of the use of a standard native fish model to investigate the effects of anti-androgens in Australian riverine environment.

Much of the overseas research in aquatic ecotoxicology has focussed on the fathead minnows, zebrafish, medaka and three-spined stickleback. These fish are not native to Australia. The effects seen in these introduced species cannot be extrapolated to Australian environment. Although, it has been stated that the hormone action in vertebrates is highly conserved, organisms are highly variable in responding to the external environment like introduction of EDCs because their biological responses are dependent on a range of intrinsic factors like species, age, nutrition, health, target tissues etc. To create reliable data for the effects of anti-androgens under Australian conditions, a native fish species needs to be examined as a test model.

The Murray rainbowfish (*Melanotaenia fluviatilis*) (family Melanotaeniidae) is a native freshwater fish species that is native to Australia. The fish reside in the Murray-Darling river system in New South Wales, Victoria and South Australia and spawn multiple times in the dry season (Pusey et al., 2001). The embryonic development is completed in 7 – 9 days at 25°C (Reid and Holdway 1995). The larvae are 3 – 5 mm at the time of hatching (Crowley et al., 1986) and display sexual dimorphism in 6 – 8 months. The adults of this fish species are easy to maintain in laboratory conditions due to their small size (< 4 g and 5 – 6 cm).

Murray Darling river basin, the natural habitat of this fish in Australia, receives treated effluents from the WWTPs. However, there have been no studies to identify/quantify EDCs, if any, in the receiving environment of Murray Darling river basin. To date, no information is available on the extent of endocrine disruption, if any, in wild Murray rainbowfish. However, it has been widely demonstrated in field studies that discharges of EDCs to aquatic ecosystems from WWTP effluents can result in adverse impacts on aquatic organisms such as fish (Tyler and Routledge 1998, Nakari 2004). It is therefore, important to investigate the sensitivity of Murray rainbowfish to environmental chemicals in robust laboratory experiments before conducting field studies using wild fish. In previous studies in our laboratory and elsewhere in Australia, sexually mature Murray rainbowfish has been effectively used as a model fish species to assess the effects of environmental estrogens (Pollino and Holdway 2002; Shanthanagouda et al., 2013; Woods and Kumar 2011). However, there is a need determine the use of Murray rainbowfish as a sensitive model to assess effects via ARs of EDCs.

1.5.6 Validation of the effects of a reference anti-androgen in fresh water fish in Australia

The classic, commercial anti-androgen flutamide has no environmental significance since it has not been detected in the aquatic environment to date. However, because of the "pure" anti-androgenic nature of flutamide, the *in vitro* anti-androgenic activity in the aquatic environment worldwide is reported in flutamide equivalents (FEQ/L). The mammalian anti-androgens, phthalates have been detected in the rivers of South Africa at concentrations ranging between 10 – 80 mg/L (Fatoki et al., 2010; Ogunfowokan et al., 2006). Anti-androgenic activity of 935 µg FEQ/L has been detected in the surface water in China (Zhao et al., 2011). A recent study in China reported that more than 1800 mg FEQ anti-androgens flow

into the gray water every day (Ma et al., 2013). *In vitro* anti-androgenic activity (370 – 4223 µg FEQ/L) has also been determined in the fresh water in Italy (Urbatzka et al., 2007). A 21-day exposure to WWTP effluent containing 328.56 µg FEQ/L of anti-androgenic activity adversely affected the reproductive behaviour of male stickleback (Sebire et al., 2011). This, therefore, necessitates the establishment of the biological effects of flutamide in laboratory conditions in Australian native fish species.

1.6. HYPOTHESES

Based on the literature review conducted on the biological effects of phthalates and flutamide, the following are the hypotheses of the present study –

1.6.1 Null hypotheses

The present study was designed to test the following hypotheses –

a) The response to short-term exposures to biologically active concentrations of flutamide is equal to the background response in unexposed sexually mature Murray rainbowfish (*Melanotaenia fluviatilis*) under laboratory exposures.

b) Short-term exposures to biologically active concentrations of flutamide elicit similar responses in sexually mature, adult male and female rainbowfish.

c) Exposures to the commercial anti-androgen, flutamide, alone and in combination, with the reference estrogen, E2 have no effect on the reproductive endocrine function and sexual development of juvenile Murray rainbowfish.

d) The response to short-term exposures of sub-acute concentrations of DnBP is equal to the background response in unexposed sexually mature Murray rainbowfish under laboratory exposures.

e) Short-term exposures to sub-acute concentrations of DnBP elicit similar responses in sexually mature, adult male and female rainbowfish.

f) Chronic exposures to environmentally relevant concentrations of DnBP have no effect on the sexual differentiation or reproductive endocrine function in juvenile Murray rainbowfish.

1.6.2 Alternate hypotheses

The following alternate hypotheses are proposed for the present study –

a) The response to short-term exposures to biologically active concentrations of flutamide is not equal to the background response in unexposed sexually mature Murray rainbowfish under laboratory exposures.

b) Short-term exposures to biologically active concentrations of flutamide elicit different responses in sexually mature, adult male and female rainbowfish.

c) Exposures to the commercial anti-androgen, flutamide, alone and in combination, with the reference estrogen, E2 affect the reproductive endocrine function and sexual development of juvenile Murray rainbowfish.

d) The response to short-term exposures to sub-acute concentrations of DnBP is not equal to the background response in unexposed sexually mature Murray rainbowfish under laboratory exposures.

e) Short-term exposures to sub-acute concentrations of DnBP elicit different responses in sexually mature, adult male and female rainbowfish.

f) Chronic exposures to environmentally relevant concentrations of DnBP affect the sexual differentiation or reproductive endocrine function in juvenile Murray rainbowfish.

1.7 AIMS

The experiments for the present project were designed to fill in the gaps in the aquatic ecotoxicology research. Figure 1 represents the outline of the structure of the thesis.

1. To investigate and validate the effects of exposures to biologically active concentrations of the commercial anti-androgen, flutamide for a short duration on the biomarkers of reproduction in sexually mature male Murray rainbowfish (Chapter 2).
2. To investigate and validate the effects of exposures to biologically active concentrations of the commercial anti-androgen, flutamide for a short duration on the biomarkers of reproduction in sexually mature female Murray rainbowfish (Chapter 3).
3. To determine the effects of the model anti-androgen, flutamide, on 17β-estradiol-induced effects in sexually undifferentiated, juvenile Murray rainbowfish (Chapter 4).

4. To investigate the effects of exposures to sub-acute concentrations of an industrial pollutant and mammalian anti-androgen, di-n-butyl phthalate for a short duration on the biomarkers of reproduction in sexually mature male Murray rainbowfish (Chapter 5).
5. To investigate the effects of exposures to sub-acute concentrations of an industrial pollutant and mammalian anti-androgen, di-n-butyl phthalate for a short duration on the biomarkers of reproduction in sexually mature female Murray rainbowfish (Chapter 6).
6. To investigate the effects of chronic exposures to environmentally relevant concentrations of di-n-butyl phthalate on gonadal histology and vitellogenin concentrations in sexually undifferentiated, juvenile rainbowfish (Chapter 7).

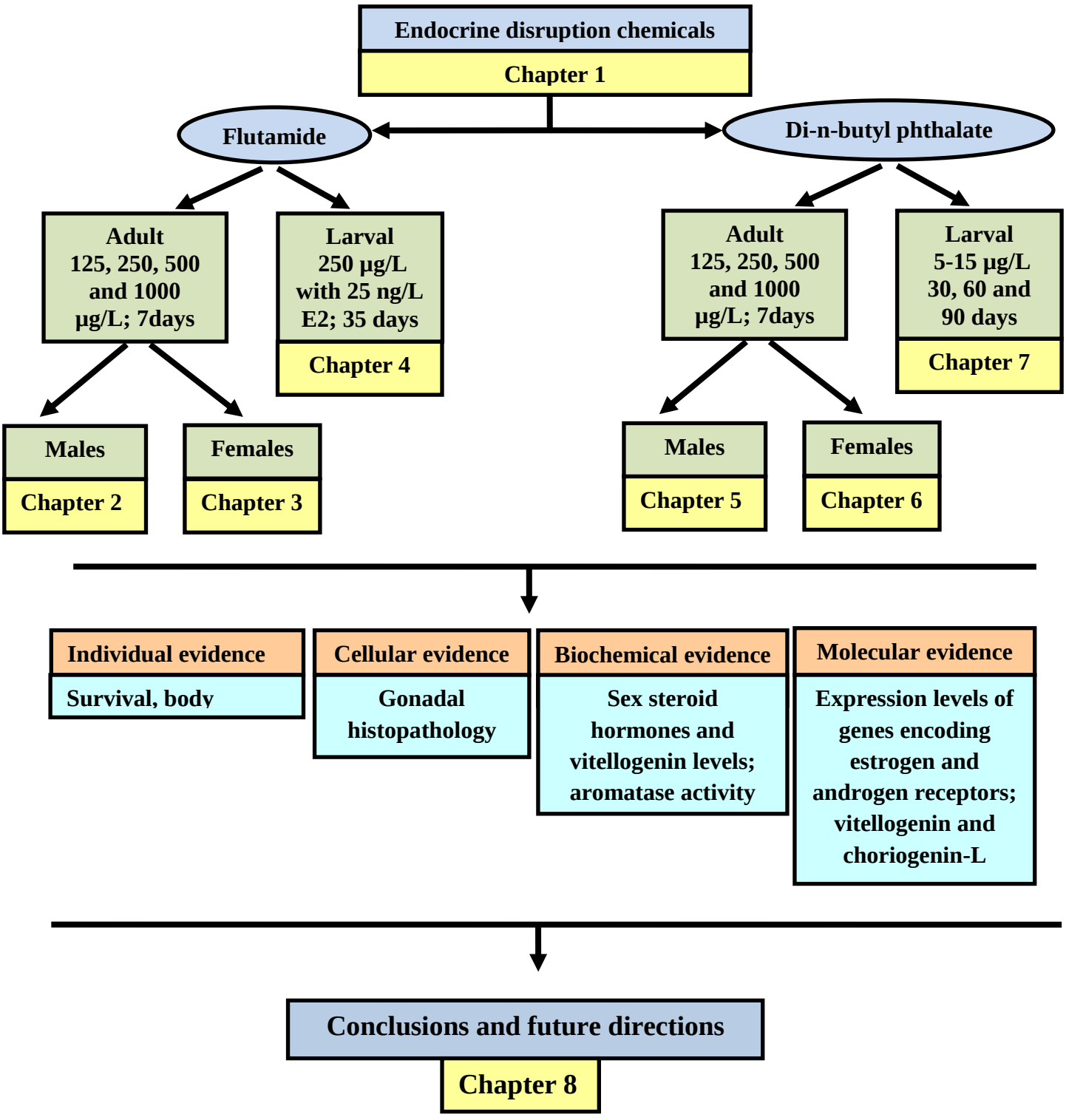


Figure 1: Flow-chart depicting thesis structure

1.8 References

Adams, W. J., Biddinger, G. R., Robillard, K. A. and Gorsuch, J. W. (1995). A summary of the acute toxicity of 14 phthalate-esters to representative aquatic organisms. Environmental Toxicology and Chemistry 14: 1569-1574.

Adebayo, O. L., James, A., Kasim, S. B. and Jagri, O. P. (2014). Leaf extracts of *Vernonia amygdalina del*. From northern Ghana contain bioactive agents that inhibit the growth of some beta-lactamase producing bacteria *in vitro*. British Journal of Pharmaceutical Research 4: 192-202.

Akerblom, N., Olsson, K., Berg, A. H., Andersson, P. L., Tysklind, M., Forlin, L. and Norrgren, L. (2000). Impact of polychlorinated naphthalenes (PCNS) in juvenile baltic salmon, *Salmo salar*: Evaluation of estrogenic effects, development, and CYP1a induction. Archives of Environmental Contamination and Toxicology 38: 225-233.

Alam, S. and Kawsar, A. (1998). Effect of estrogens on growth and sex-ratio in the genetically improved farmed tilapia, *Oreochromis niloticus* (L.). Bangladesh Journal of Zoology 26: 37-43.

Allen, Y., Matthiessen, P., Scott, A. P., Haworth, S., Feist, S. and Thain, J. E. (1999). The extent of oestrogenic contamination in the UK estuarine and marine environments-further surveys of flounder. Science of Total Environment 233: 5-20.

Almstrup, K., Fernandez, M. F., Petersen, J. H., Olea, N., Skakkebaek, N. E. and Leffers, H. (2002). Dual effects of phytoestrogens result in U-shaped dose-response curves. Environmental Health Perspectives 110: 743-748.

Alzieu, C. (2000). Impact of tributyltin on marine invertebrates. Ecotoxicology 9: 71-76.

Anand-Ivell, R., Heng, K., Hafen, B., Setchell, B. and Ivell, R. (2009). Dynamics of INSL3 peptide expression in the rodent testis. Biology of Reproduction 81: 480-487.

Andersen, H. R., Andersson, A.-M., Arnold, S. F., Autrup, H., Barfoed, M., Beresford, N. A., Bjerregaard, P., Christiansen, L. B., Gissel, B., Hummel, R., Jorgensen, E. B., Korsgaard, B., Guevel, R. L., Leffers, H., McLachlan, J., Moller, A., Nielsen, J. B., Olea, N., Oles-Karasko, A., Pakdel, F., Pedersen, K. L., Perez, P., Skakkeboek, N. E., Sonnenschein, C., Soto, A. M., Sumpter, J. P., Thorpe, S. M. and Grandjean, P. (1999). Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. Environmental Health Perspectives 107: 89-108.

Andersen, L., Bjerregaard, P. and Korsgaard, B. (2003). Vitellogenin induction and brain aromatase activity in adult male and female zebrafish exposed to endocrine disrupters. Fish Physiology and Biochemistry 28: 319-321.

Ankley, G. T., Defoe, D. L., Kahl, M. D., Jensen, K. M., Miracle, A., Hartig, P., Gray, L. E., Cardon, M. and Wilson, V. (2004). Evaluation of the model anti-androgen flutamide for assessing the mechanistic basis of responses to an androgen in the fathead minnow (*Pimephales promelas*). Environmental Science and Technology 38: 6322-6327.

Ankley, G. T., Jensen, K. M., Kahl, M. D., Durhan, E. J., Makynen, E. A., Cavallin, J. E., Martinovic, D., Wehmas, L. C., Mueller, N. D. and Villeneuve, D. L. (2010). Use of chemical mixtures to differentiate mechanisms of endocrine action in a small fish model. Aquatic Toxicology 99: 389-396.

Annammala, K. V., Abdullah, M. H., Bin Mokhtar, M., Joseph, C. G. and Sakari, M. (2013). Characterization of aromatic hydrocarbons in tropical coastal water of Sabah, Borneo. Asian Journal of Chemistry 25: 3773-3780.

Anway, M. D., Rekow, S. S. and Skinner, M. K. (2008). Comparative anti-androgenic actions of vinclozolin and flutamide on transgenerational adult onset disease and spermatogenesis. Reproductive Toxicology 26: 100-106.

Aoki, K. A. A., Harris, C. A., Katsiadaki, I. and Sumpter, J. P. (2011). Evidence suggesting that di-n-butyl phthalate has antiandrogenic effects in fish. Environmental Toxicology and Chemistry 30: 1338-1345.

Arcaro, K. F., O'Keefe, P. W., Yang, Y., Clayton, W. and Gierthy, J. F. (1999). Antiestrogenicity of environmental polycyclic aromatic hydrocarbons in human breast cancer cells. Toxicology 133: 115-127.

Arias, E. (2012). Effects of the peroxisome proliferator di(2-ethylhexyl)phthalate on cell turnover and peroxisome proliferation in primary chick embryo hepatocytes. Environmental Toxicology and Chemistry 31: 2856-2860.

Arukwe, A., Celius, T., Walther, B. T. and Goksoyr, A. (2000). Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (*Salmo salar*). Aquatic Toxicology 49: 159-170.

Asai, D., Tahara, Y., Nakai, M., Yakabe, Y., Takatsuki, M., Nose, T., Shinmyozu, T. and Shimohigashi, Y. (2000). Structural essentials of xenoestrogen dialkyl phthalates to bind to the estrogen receptors. Toxicology Letters 118: 1-8.

Autian, J. (1973). Toxicity and health threats of phthalate esters: Review of the literature. Environmental Health Perspectives: 3-26.

Baatrup, E. and Junge, M. (2001). Antiandrogenic pesticides disrupt sexual characteristics in the adult male guppy (*Poecilia reticulata*). Environmental Health Perspectives 109: 1063-1070.

Baker, V. A. (2001). Endocrine disrupters - testing strategies to assess human hazard. Toxicology *in vitro* 15: 413-419.

Balch, G. and Metcalfe, C. (2006). Developmental effects in Japanese medaka (*Oryzias latipes*) exposed to nonylphenol ethoxylates and their degradation products. Chemosphere 62: 1214-1223.

Baldwin, W. S., Milam, D. L. and LeBlanc, G. A. (1995). Physiological and biochemical perturbations in *Daphnia magna* following exposure to the model environmental estrogen diethylstilbestrol. Environmental Toxicology and Chemistry 14: 945-952.

Banerjee, R., Pathmasiri, W., Snyder, R., McRitchie, S. and Sumner, S. (2012). Metabolomics of brain and reproductive organs: Characterizing the impact of gestational exposure to butylbenzyl phthalate on dams and resultant offspring. Metabolomics 8: 1012-1025.

Barco-Bonilla, N., Plaza-Bolanos, P., Valera Tarifa, N. M., Romero-Gonzalez, R., Martinez Vidal, J. L. and Garrido Frenich, A. (2014). Highly sensitive determination of polybrominated diphenyl ethers in surface water by GC coupled to high-resolution MS according to the EU water directive. Journal of Separation Science 37: 69-76.

Barnhoorn, I. E., Bornman, M. S., Pieterse, G. M. and van Vuren, J. H. (2004). Histological evidence of intersex in feral sharptooth catfish (*Clarias gariepinus*) from an estrogen-polluted water source in Gauteng, South Africa. Environmental Toxicology 19: 603-608.

Barr, D. B., Silva, M. J., Kato, K., Reidy, J. A., Malek, N. A., Hurtz, D., Sadowski, M., Needham, L. L. and Calafat, A. M. (2003). Assessing human exposure to phthalates using monoesters and their oxidized metabolites as biomarkers. Environment Health Perspectives 111: 1148-1151.

Barse, A. V., Chakrabarti, T., Ghosh, T. K., Pal, A. K. and Jadhao, S. B. (2007). Endocrine disruption and metabolic changes following exposure of *Cyprinus carpio* to diethyl phthalate. Pesticide Biochemistry and Physiology 88: 36-42.

Batty, J. and Lim, R. (1999). Morphological and reproductive characteristics of male mosquitofish (*Gambusia affinis holbrooki*) inhabiting sewage-contaminated waters in New South Wales, Australia. Archives of Environmental Contamination Toxicology 36: 301-307.

Bauer, M., Greenwood, S. J., Clark, K. F., Jackman, P. and Fairchild, W. (2013). Analysis of gene expression in *Homarus americanus* larvae exposed to sublethal concentrations of endosulfan during metamorphosis. Comparative Biochemistry and Physiology 8: 300-308.

Bayley, M., Junge, M. and Baatrup, E. (2002). Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquatic Toxicology 56: 227-239.

Beaven, U. and Muposhi, V. (2012). Aspects of a monosex population of *Oreochromis niloticus* fingerlings produced using 17-alpha methyl testosterone hormone. Journal of Aquaculture Research and Development 3: 132-132.

Belfroid, A. C., Van der Horst, A., Vethaak, A. D., Schafer, A. J., Rijs, G. B. J., Wegener, J. and Cofino, W. P. (1999). Analysis and occurence of estrogenic hormones and their glucuronides in surface water and waste water in the Netherlands. Science of the Total Environment 225: 101-108.

Ben Salem, Z., Capelli, N., Grisey, E., Baurand, P.-E., Ayadi, H. and Aleya, L. (2014). First evidence of fish genotoxicity induced by heavy metals from landfill leachates: The advantage of using the RAPD-PCR technique. Ecotoxicology and Environmental Safety 101: 90-96.

Billiard, S. M., Bols, N. C. and Hodson, P. V. (2004). *In vitro* and *in vivo* comparisons of fish-specific CYP1a induction relative potency factors for selected polycyclic aromatic hydrocarbons. Ecotoxicology and Environmental Safety 59: 292-299.

Bjerregaard, L. B., Korsgaard, B. and Bjerregaard, P. (2006). Intersex in wild roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams. Ecotoxicology and Environmental Safety 64: 321-328.

Blount, B. C., Silva, M. J., Caudill, S. P., Needham, L. L., Pirkle, J. L., Sampson, E. J., Lucier, G. W., Jackson, R. J. and Brock, J. W. (2000). Levels of seven urinary phthalate metabolites in a human reference population. Environmental Health Perspectives 108: 979-982.

Blum, J. L., Nyagode, B. A., James, M. O. and Denslow, N. D. (2008). Effects of the pesticide methoxychlor on gene expression in the liver and testes of the male largemouth bass (*Micropterus salmoides*). Aquatic Toxicology 86: 459-469.

Bones, J., Thomas, K. V. and Paull, B. (2006). Improved method for the determination of zinc pyrithione in environmental water samples incorporating on-line extraction and

preconcentration coupled with liquid chromatography atmospheric pressure chemical ionisation mass spectrometry. Journal of Chromatography 1132: 157-164.

Borg, B. (1994). Androgens in teleost fishes. Comparative Biochemistry and Physiology C-Pharmacology Toxicology and Endocrinology 109: 219-245.

Brand, T. and Slob, A. K. (1991). Perinatal flutamide and mounting and lordosis behavior in adult female wistar and sprague-dawley rats. Behavioural Brain Research 44: 43-51.

Brion, F., Tyler, C. R., Palazzi, X., Laillet, B., Porcher, J. M., Garric, J. and Flammarion, P. (2004). Impacts of 17β-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo, larval, juvenile and adult life stages in zebrafish (*Danio rerio*). Aquatic Toxicology 68: 193-217.

Brockmeier, E. K., Ogino, Y., Iguchi, T., Barber, D. S. and Denslow, N. D. (2013). Effects of 17beta-trenbolone on eastern and western mosquitofish (*Gambusia holbrooki* and *Gambusia affinis*) anal fin growth and gene expression patterns. Aquatic Toxicology 128: 163-170.

Brown, R. J., Conradi, M. and Depledge, M. H. (1999). Long-term exposure to 4-nonylphenol affects sexual differentiation and growth of the amphipod *Corophium volutator* (Pallas, 1766). Science of the Total Environment 233: 77-88.

Buccafusco, R. J., Ells, S. J. and Leblanc, G. A. (1981). Acute toxicity of priority pollutants to bluegill (*Lepomis macrochirus*). Bulletin of Environmental Contamination and Toxicology 26: 446-452.

Campinho, M. A. and Power, D. M. (2013). Waterborne exposure of zebrafish embryos to micromole concentrations of ioxynil and diethylstilbestrol disrupts thyrocyte development. Aquatic Toxicology 140: 279-287.

Carlson, D. B. and Williams, D. E. (1999). Sex-specific vitellogenin production in immature rainbow trout. Environmental Toxicology and Chemistry 18: 2361-2363.

Carnevali, O., Tosti, L., Speciale, C., Peng, C., Zhu, Y. and Maradonna, F. (2010). DEHP impairs zebrafish reproduction by affecting critical factors in oogenesis. Plos One 5(4): e10201.

Celius, T., Matthews, J. B., Giesy, J. P. and Zacharewski, T. R. (2000). Quantification of rainbow trout (*Oncorhynchus mykiss*) zona radiata and vitellogenin mRNA levels using real-time PCR after *in vivo* treatment with estradiol-17 beta or alpha-zearalenol. Journal of Steroid Biochemistry and Molecular Biology 75: 109-119.

Chandolia, R. K., Weinbauer, G. F., Simoni, M., Behre, H. M. and Nieschlag, E. (1991). Comparative effects of chronic administration of the nonsteroidal antiandrogens

flutamide and casodex on the reproductive-system of the adult male-rat. Acta Endocrinologica 125: 547-555.

Chauvigne, F., Plummer, S., Lesne, L., Cravedi, J. P., Dejucq-Rainsford, N., Fostier, A. and Jegou, B. (2011). Mono-(2-ethylhexyl) phthalate directly alters the expression of leydig cell genes and CYP17 lyase activity in cultured rat fetal testis. Plos One 6(11): e27172.

Chen, P. S., Haung, W. Y. and Huang, S. D. (2014). Analysis of triazine herbicides using an up-and-down-shaker-assisted dispersive liquid-liquid microextraction coupled with gas chromatography-mass spectrometry. Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences 955-956: 116-123.

Chikae, M., Ikeda, R., Hasan, Q., Morita, Y. and Tamiya, E. (2004a). Effects of tamoxifen, 17a-ethynylestradiol, flutamide, and methyltestosterone on plasma vitellogenin levels of male and female Japanese medaka (*Oryzias latipes*). Environmental Toxicology and Pharmacology 17: 29-33.

Chikae, M., Hatano, Y., Ikeda, R., Morita, Y., Hasan, Q. and Tamiya, E. (2004b). Effects of bis(2-ethylhexyl) phthalate and benzopyrene on the embryos of Japanese medaka (*Oryzias latipes*). Environmental Toxicology and Pharmacology 16: 141-145.

Chikae, M., Ikeda, R., Hatano, Y., Hasan, Q., Morita, Y. and Tamiya, E. (2004c). Effects of bis(2-ethylhexyl) phthalate, gamma-hexachlorocyclohexane, and 17 beta-estradiol on the fry stage of medaka (*Oryzias latipes*). Environmental Toxicology and Pharmacology 18: 9-12.

Cho, H. K., Kong, H. J., Kim, H. Y. and Cheong, J. (2012). Characterization of *Paralichthys olivaceus* peroxisome proliferator-activated receptor-alpha gene as a master regulator of flounder lipid metabolism. General and Comparative Endocrinology 175: 39-47.

Chow, W. S., Chan, W. K. L. and Chan, K. M. (2013). Toxicity assessment and vitellogenin expression in zebrafish (*Danio rerio*) embryos and larvae acutely exposed to bisphenol A, endosulfan, heptachlor, methoxychlor and tetrabromobisphenol A. Journal of Applied Toxicology 33: 670-678.

Christiansen, L. B., Pedersen, K. L., Pedersen, S. N., Korsgaard, B. and Bjerregaard, P. (2000). *In vivo* comparison of xenoestrogens using rainbow trout vitellogenin induction as a screening system. Environmental Toxicology and Chemistry 19: 1867-1874.

Colborn, T. (1996). Our stolen future - book review. Journal of Cleaner Production 4: 123-124.

Collins, B. M., Mclachlan, J. A. and Arnold, S. F. (1997). The estrogenic and antiestrogenic activities of phytochemicals with human estrogen receptor expressed in yeast. Steroids 123: 365-372.

Cousins, I. T., Mackay, D. and Parkerton, T. F. (2003). Physical-chemical properties and evaluative fate modelling of phthalate esters. Phthalate Esters 3: 57-84.

Crago, J. and Klaper, R. (2012). A mixture of an environmentally realistic concentration of a phthalate and herbicide reduces testosterone in male fathead minnow (*Pimephales promelas*) through a novel mechanism of action. Aquatic toxicology (Amsterdam, Netherlands) 110-111: 74-83.

Cravedi, J. P. and Perdu-Durand, E. (2002). The phthalate diesters DEHP and DBP do not induce lauric acid hydroxylase activity in rainbow trout. Marine Environmental Research 54: 787-791.

Crews, D., Bergeron, J. M. and McLachlan, J. A. (1995). The role of estrogen in turtle sex determination and the effect of PCBS. Environmental Health Perspectives 103: 73-77.

Crowley, L., Ivantsoff, W. and Allen, G. R. (1986). Taxonomic position of 2 crimson-spotted rainbowfish, *Melanotaenia duboulayi* and *Melanotaenia fluviatilis* (Pisces, Melanotaeniidae), from eastern Australia, with special reference to their early life-history stages. Australian Journal of Marine and Freshwater Research 37: 385-398.

Csaba, G. (2013). Hormonal imprinting in the central nervous system: Causes and consequences. Orvosi Hetilap 154: 128-135.

Da Cuna, R. H., Pandolfi, M., Genovese, G., Piazza, Y., Ansaldo, M. and Lo Nostro, F. L. (2013). Endocrine disruptive potential of endosulfan on the reproductive axis of *Cichlasoma dimerus* (Perciformes, Cichlidae). Aquatic Toxicology 126: 299-305.

Danzo, B. J. (1998). The effects of environmental hormones on reproduction. Cellular and Molecular Life Sciences 54: 1249-1264.

Dargnat, C., Blanchard, M., Chevreuil, M. and Teil, M. J. (2009). Occurrence of phthalate esters in the Seine river estuary (France). Hydrological Processes 23: 1192-1201.

Dasgupta, S., Banerjee, K., Utture, S., Kusari, P., Wagh, S., Dhumal, K., Kolekar, S. and Adsule, P. G. (2011). Extraction of pesticides, dioxin-like PCBs and PAHs in water based commodities using liquid-liquid microextraction and analysis by gas chromatography-mass spectrometry. Journal of Chromatography 1218: 6780-6791.

Davis, B. J., Maronpot, R. R. and Heindel, J. J. (1994). Di-(2-ethylhexyl) phthalate suppresses estradiol and ovulation in cycling rats. Toxicology and Applied Pharmacology 128: 216-223.

Davis, L. K., Fox, B. K., Lim, C., Hiramatsu, N., Sullivan, C. V., Hirano, T. and Grau, E. G. (2009). Induction of vitellogenin production in male tilapia (*Oreochromis mossambicus*) by commercial fish diets. Comparative Biochemistry and Physiology 154: 249-254.

de Jourdan, B. P., Hanson, M. L., Muir, D. C. G. and Solomon, K. R. (2013). Environmental fate of three novel brominated flame retardants in aquatic mesocosms. Environmental Toxicology and Chemistry 32: 1060-1068.

de Lafontaine, Y., Beauvais, C., Cessna, A. J., Gagnon, P., Hudon, C. and Poissant, L. (2014). Sulfonylurea herbicides in an agricultural catchment basin and its adjacent wetland in the St. Lawrence river basin. Science of Total Environment 479-480: 1-10.

de Solla, S. R., Bishop, C. A. and Brooks, R. J. (2002). Sexually dimorphic morphology of hatchling snapping turtles (*Chelydra serpentina*) from contaminated and reference sites in the great lakes and St Lawrence river basin, North America. Environmental Toxicology and Chemistry 21: 922-929.

Delaere, K. P. J. and Vanthillo, E. L. (1991). Flutamide monotherapy as primary-treatment in advanced prostatic-carcinoma. Seminars in Oncology 18: 13-18.

Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P. and Waldock, M. (1998). Identification of estrogenic chemicals in STW effluent - 1 - chemical fractionation and *in-vitro* biological screening. Environmental Science and Technology 33: 1549-1558.

Dong, M., Zhu, L., Shao, B., Zhu, S., Wang, J., Xie, H., Wang, J. and Wang, F. (2013). The effects of endosulfan on cytochrome p450 enzymes and glutathione-S-transferases in zebrafish (*Danio rerio*) livers. Ecotoxicology and Environmental Safety 92: 1-9.

Doyle, T. J., Bowman, J. L., Windell, V. L., McLean, D. J. and Kim, K. H. (2013). Transgenerational effects of di-(2-ethylhexyl) phthalate on testicular germ cell associations and spermatogonial stem cells in mice. Biology of Reproduction 88(5): 1-15.

Duag, Z., Wang, H., Li, N., Wang, L. and Zhu, L. (2013). Effects of bisphenol A on the transcriptions of typical functional genes in adult zebrafish (*Danio rerio*) liver. Asian Journal of Ecotoxicology 8: 931-936.

Durlej, M., Knapczyk-Stwora, K. and Slomczynska, M. (2012). Prenatal and neonatal flutamide administration increases proliferation and reduces apoptosis in large antral follicles of adult pigs. Animal Reproduction Science 132: 58-65.

Ema, M., Miyawaki, E. and Kawashima, K. (2000). Critical period for adverse effects on development of reproductive system in male offspring of rats given di-n-butyl phthalate during late pregnancy. Toxicology Letters 111: 271-278.

Endo, T., Todo, T., Lokman, P. M., Kudo, H., Ijiri, S., Adachi, S. and Yamauchi, K. (2011). Androgens and very low density lipoprotein are essential for the growth of previtellogenic oocytes from Japanese eel, *Anguilla japonica*, *in vitro*. Biology of Reproduction 84: 816-825.

Estevez, E., del Carmen Cabrera, M., Molina-Diaz, A., Robles-Molina, J. and del Pino Palacios-Diaz, M. (2012). Screening of emerging contaminants and priority substances in reclaimed water for irrigation and groundwater in a volcanic aquifer (*Gran canaria*, Canary Islands, Spain). Science of the Total Environment 433: 538-546.

Falahatkar, B., Poursaeid, S., Meknatkhah, B., Khara, H. and Efatpanah, I. (2014). Long-term effects of intraperitoneal injection of estradiol-17 beta on the growth and physiology of juvenile stellate sturgeon *Acipenser stellatus*. Fish Physiology and Biochemistry 40: 365-373.

Fatoki, O. S. and Vernon, F. (1990). Phthalate-esters in rivers of the Greater Manchester area, UK. Science of the Total Environment 95: 227-232.

Fatoki, O. S. and Ogunfowokan, A. O. (1993). Determination of phthalate ester plasticizers in the aquatic environment of Southwestern Nigeria. Environment International 19: 619-623.

Fatoki, O. S. and Noma, A. (2001). Determination of phthalate esters in the aquatic environment. South African Journal of Chemistry 54: 1-15.

Fatoki, O. S. and Noma, A. (2002). Solid phase extraction method for selective determination of phthalate esters in the aquatic environment. Water Air and Soil Pollution 140: 85-98.

Fatoki, O. S., Bornman, M., Ravandhalala, L., Chimuka, L., Genthe, B. and Adeniyi, A. (2010). Phthalate ester plasticizers in freshwater systems of Venda, South Africa and potential health effects. Water Safety 36: 117-125.

Fau, D., Eugene, D., Berson, A., Letteron, P., Fromenty, B., Fisch, C. and Pessayre, D. (1994). Toxicity of the antiandrogen flutamide in isolated rat hepatocytes. Journal of Pharmacology and Experimental Therapeutics 269: 954-962.

Feldman, B. J. and Feldman, D. (2001). The development of androgen-independent prostate cancer. Nature Reviews Cancer 1: 34-45.

Fensterheim, R. J. (2001). Comment on "estrogenicity determination in sewage treatment plants and surface waters from the Catalonian area (Spain)". Environmental Science and Technology 35: 4156.

Feswick, A., Ankley, G. T., Denslow, N., Ellestad, L. E., Fuzzen, M., Jensen, K. M., Kroll, K., Lister, A., Maclatchy, D. L., McMaster, M. E., Orlando, E. F., Servos, M. R., Tetreault, G. R., Van Den Heuvel, M. R. and Munkittrick, K. R. (2014). An inter-laboratory study on the variability in measured concentrations of 17beta-estradiol, testosterone, and 11-ketotestosterone in white sucker: Implications and recommendations. Environmental Toxicology and Chemistry 33: 847-857.

Filby, A. L., Thorpe, K. L., Maack, G. and Tyler, C. R. (2007). Gene expression profiles revealing the mechanisms of anti-androgen-and estrogen-induced feminization in fish. Aquatic Toxicology 81: 219-231.

Fisher, J. S., Macpherson, S., Marchetti, N. and Sharpe, R. M. (2003). Human 'testicular dysgenesis syndrome': A possible model using *in utero* exposure of the rat to dibutyl phthalate. Human Reproduction 18: 1383-1394.

Flynn, K., Swintek, J. and Johnson, R. (2013). Use of gene expression data to determine effects on gonad phenotype in Japanese medaka after exposure to trenbolone or estradiol. Environmental Toxicology and Chemistry 32: 1344-1353.

Folmar, L. C., Denslow, N. D., Kroll, K., Orlando, E. F., Enblom, J., Marcino, J., Metcalfe, C. and Guillette, L. J., Jr. (2001). Altered serum sex steroids and vitellogenin induction in walleye (*Stizostedion vitreum*) collected near a metropolitan sewage treatment plant. Archives of Environmental Contamination and Toxicology 40: 392-398.

Frederiksen, H., Aksglaede, L., Sorensen, K., Skakkebaek, N. E., Juul, A. and Andersson, A. M. (2011). Urinary excretion of phthalate metabolites in 129 healthy Danish children and adolescents: Estimation of daily phthalate intake. Environmental Research 111: 656-663.

Fromme, H., Kuchler, T., Otto, T., Pilz, K., Muller, J. and Wenzel, A. (2002). Occurrence of phthalates and bisphenol A and F in the environment. Water Research 36: 1429-1438.

Fujita, T., Fukada, H., Shimizu, M., Hiramatsu, N. and Hara, A. (2004). Quantification of serum levels of precursors to vitelline envelope proteins (choriogenins) and vitellogenin in estrogen treated masu salmon, *Oncorhynchus masou*. General and Comparative Endocrinology 136: 49-57.

Gazo, I., Linhartova, P., Shaliutina, A. and Hulak, M. (2013). Influence of environmentally relevant concentrations of vinclozolin on quality, DNA integrity, and antioxidant responses of sterlet *Acipenser ruthenus* spermatozoa. Chemico-Biological Interactions 203: 377-385.

Gercken, J. and Sordyl, H. (2002). Intersex in feral marine and freshwater fish from northeastern Germany. Marine Environ Research 54: 651-5.

Ghorpade, N., Mehta, V., Khare, M., Sinkar, P., Krishnan, S. and Rao, C. V. (2002). Toxicity study of diethyl phthalate on freshwater fish *Cirrhina mrigala*. Ecotoxicology and Environmental Safety 53: 255-258.

Gimeno, S., Komen, H., Gerritsen, A. G. M. and Bowmer, T. (1998). Feminisation of young males of the common carp, *Cyprinus carpio*, exposed to 4-*tert*-pentylphenol during sexual differentiation. Environmental Toxicology and Chemistry 43: 77-92.

Glover, C. N., Petri, D., Tollefsen, K.-E., Jorum, N., Handy, R. D. and Berntssen, M. H. G. (2007). Assessing the sensitivity of atlantic salmon (*Salmo salar*) to dietary endosulfan exposure using tissue biochemistry and histology. Aquatic Toxicology 84: 346-355.

Gong, X., Xu, Y., Ren, L., Zhang, S., Zhang, X. and Zhang, L. (2010). Estrogenic effects of octylphenol on carp (*Cyprinus carpio*). Journal of Fisheries of China 34: 410-414.

Gooding, M. P., Wilson, V. S., Folmar, L. C., Marcovich, D. T. and LeBlanc, G. A. (2003). The biocide tributyltin reduces the accumulation of testosterone as fatty acid esters in the mud snail (*Ilyanassa obsoleta*). Environmental Health Perspectives 111: 426-430.

Goto, K., Koizumi, K., Takaori, H., Fujii, Y., Furuyama, Y., Saika, O., Suzuki, H., Saito, K. and Suzuki, K. (2004). Effects of flutamide on sex maturation and behavior of offspring born to female rats treated during late pregnancy. Journal of Toxicological Sciences 29: 517-34.

Graham, P. R. (1973). Phthalate ester plasticizers-why and how they are used. Environmental Health Perspectives 3: 3-12.

Grande, S. W., Andrade, A. J. M., Talsness, C. E., Grote, K., Golombiewski, A., Sterner-Kock, A. and Chahoud, I. (2007). A dose-response study following *in utero* and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): Reproductive effects on adult female offspring rats. Toxicology 229: 114-122.

Gravina, L., Ruiz, F., Bidaurrazaga, I., Zabala, J., Badiola, A., Granados, C., Lekue, J. A., Irazusta, J. and Gil, S. M. (2013). Influence of dietary phytosteroids in the antioxidant

capacity in elite and sub-elite soccer players. Annals of Nutrition and Metabolism 63: 790-790.

Gray, M. A. and Metcalfe, C. D. (1997). Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to p-nonylphenol. Environmental Toxicology and Chemistry 16: 1082-1086.

Gray, M. A., Niimi, A. J. and Metcalfe, C. D. (1999). Factors affecting the development of testis-ova in medaka, *Oryzias latipes*, exposed to octylphenol. Environmental Toxicology and Chemistry 18: 1835-1842.

Green, C., Williams, R., Kanda, R., Churchley, J., He, Y., Thomas, S., Goonan, P., Kumar, A. and Jobling, S. (2013). Modeling of steroid estrogen contamination in UK and South Australian rivers predicts modest increases in concentrations in the future. Environmental Science and Technology 47: 7224-7232.

Grist, E. P. M., Wells, N. C., Whitehouse, P., Brighty, G. and Crane, M. (2003). Estimating the effects of 17 alpha-ethinylestradiol on populations of the fathead minnow *Pimephales promelas*: Are conventional toxicological endpoints adequate? Environmental Science and Technology 37: 1609-1616.

Ha, C. R. and Iuchi, I. (1997). Purification and partial characterization of 76 kda transglutaminase in the egg envelope (chorion) of rainbow trout, *Oncorhynchus mykiss*. Journal of Biochemistry 122: 947-954.

Han, Z.-X., Lv, C.-X. and Li, H. (2009). Effects of bis(2-ethylhexyl) phthalate on sex hormones of common carp (*Cyprinus carpio*) and the protection of zinc. Synthesis and Reactivity in Inorganic Metal-Organic and Nano-Metal Chemistry 39: 100-105.

Hanselman, T. A., Graetz, D. A. and Wilkie, A. C. (2003). Manure-borne estrogens as potential environmental contaminants: A review. Environmental Science and Technology 37: 5471-5478.

Harrahy, E. A., Edwards, D. S. and Hedman, C. J. (2014). Persistence of 2,4-D and its effects on benthic macroinvertebrates following spring treatment of Eurasian watermilfoil, *Myriophyllum spicatum* (L.) In two lakes in southeastern Wisconsin, USA. Bulletin of Environmental Contamination and Toxicology 92: 404-409.

Harries, J. E., Runnalls, T., Hill, E., Harris, C. A., Maddix, S., Sumpter, J. P. and Tyler, C. R. (2000). Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). Environmental Science and Technology 34: 3003-3011.

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J. P., Tylor, T. and Zaman, N. (1997). Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. Environmental Toxicology and Chemistry 16: 534-542.

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Routledge, E. J., Rycroft, R., Sumpter, J. P. and Tylor, T. (1996). A survey of estrogenic activity in United Kingdom inland waters. Environmental Toxicology and Chemistry 15: 1993-2002.

Harris, C. A., Henttu, P., Parker, M. G. and Sumpter, J. P. (1997). The estrogenic activity of phthalate esters *in vitro*. Environmental Health Perspectives 105: 802-811.

Hashimoto, S., Takazawa, Y., Fushimi, A., Tanabe, K., Shibata, Y., Ieda, T., Ochiai, N., Kanda, H., Ohura, T., Tao, Q. and Reichenbach, S. E. (2011). Global and selective detection of organohalogens in environmental samples by comprehensive two-dimensional gas chromatography-tandem mass spectrometry and high-resolution time-of-flight mass spectrometry. Journal of Chromatography 1218: 3799-3810.

Hayes, T. B., Haston, K., Tsui, M., Hoang, A., Haeffele, C. and Vonk, A. (2003). Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): Laboratory and field evidence. Environmental Health Perspectives 111: 568-575.

Hayes, T. B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A. A. and Vonk, A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences of the United States of America 99: 5476-5480.

Heidler, J. and Halden, R. U. (2009). Fate of organohalogens in US wastewater treatment plants and estimated chemical releases to soils nationwide from biosolids recycling. Journal of Environmental Monitoring 11: 2207-2215.

Hejmej, A., Kopera, I., Kotula-Balak, M., Lydka, M., Lenartowicz, M. and Bilinska, B. (2012). Are expression and localization of tight and adherens junction proteins in testes of adult boar affected by foetal and neonatal exposure to flutamide? International Journal of Andrology 35: 340-352.

Helander, B., Olsson, A., Bignert, A., Asplund, L. and Litzen, K. (2002). The role of DDE, PCB, coplanar PCB and eggshell parameters for reproduction in the white-tailed sea eagle (*Haliaeetus albicilla*) in Sweden. Ambio 31: 386-403.

Hemmer, M. J., Hemmer, B. L., Bowman, C. J., Kroll, K. J., Folmar, L. C., Marcovich, D., Hoglund, M. D. and Denslow, N. D. (2001). Effects of *p*-nonylphenol, methoxychlor,

and endosulfan on vitellogenin induction and expression in sheepshead minnow (*Cyprinodon variegatus*). Environmental Toxicology and Chemistry 20: 336-343.

Heng, K., Anand-Ivell, R., Teerds, K. and Ivell, R. (2012). The endocrine disruptors dibutyl phthalate (DBP) and diethylstilbestrol (DES) influence leydig cell regeneration following ethane dimethane sulphonate treatment of adult male rats. International Journal of Andrology 35: 353-363.

Hill, E. M., Evans, K. L., Horwood, J., Rostkowski, P., Oladapo, F. O., Gibson, R., Shears, J. A. and Tyler, C. R. (2010). Profiles and some initial identifications of (anti)androgenic compounds in fish exposed to wastewater treatment works effluents. Environmental Science and Technology 44: 1137-1143.

Hodson, P. V., Qureshi, K., Noble, C. A. J., Akhtar, P. and Brown, R. S. (2007). Inhibition of cyp1a enzymes by alpha-naphthoflavone causes both synergism and antagonism of retene toxicity to rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 81: 275-285.

Hotchkiss, A. K., Ankley, G. T., Wilson, V. S., Hartig, P. C., Durhan, E. J., Jensen, K. M., Martinovi, D. and Gray, L. E. (2008). Of mice and men (and mosquitofish): Antiandrogens and androgens in the environment. Bioscience 58: 1037-1050.

Hu, G.-X., Lian, Q.-Q., Ge, R.-S., Hardy, D. O. and Li, X.-K. (2009). Phthalate-induced testicular dysgenesis syndrome: Leydig cell influence. Trends in Endocrinology and Metabolism 20: 139-145.

Huang, G. L., Sun, H. W. and Song, Z. H. (1999). Interactions between dibutyl phthalate and aquatic organisms. Bulletin of Environmental Contamination and Toxicology 63: 759-765.

Humble, J. L., Saaristo, M., Lindstrom, K., Lehtonen, K. K. and Craft, J. A. (2014). Effects of 17alpha-ethinyl estradiol exposure on estrogen receptors alpha and beta and vitellogenins a, b and c mRNA expression in the liver of sand goby (*Pomatoschistus minutus*). Marine Environmental Research 96: 12-18.

Hwang, H. M., Oh, J. R., Kahng, S. H. and Lee, K. W. (1999). Tributyltin compounds in mussels, oysters and sediments of Chinhae bay, Korea. Marine Environmental Research 47: 61-70.

Ibor, O. R., Chukwuka, A. V. and Adeogun, A. O. (2014). First evidence of endocrine disruption in tilapias from Ogun river, Nigeria. Drug Metabolism Reviews 45: 124-124.

Imajima, T., Shono, T., Zakaria, O. and Suita, S. (1997). Prenatal phthalate causes cryptorchidism postnatally by inducing transabdominal ascent of the testis in fetal rats. Journal of Pediatric Surgery 32: 18-21.

Ivell, R., Heng, K., Nicholson, H. and Anand-Ivell, R. (2013). Brief maternal exposure of rats to the xenobiotics dibutyl phthalate or diethylstilbestrol alters adult-type leydig cell development in male offspring. Asian Journal of Andrology 15: 261-268.

Jarmolowicz, S., Demska-Zakes, K. and Zakes, Z. (2013). Impact of di-n-butyl phthalate on reproductive system development in European pikeperch (*Sander lucioperca*). Acta Veterinaria Brno 82: 197-201.

Jaworska, J. S., Hunter, R. S. and Schultz, T. W. (1995). Quantitative structure-toxicity relationships and volume fraction analyses for selected esters. Archives of Environmental Contamination and Toxicology 29: 86-93.

Jee, J. H., Koo, J. G., Keum, Y. H., Park, K. H., Choi, S. H. and Kang, J. C. (2009). Effects of dibutyl phthalate and di-ethylhexyl phthalate on acetylcholinesterase activity in bagrid catfish, *Pseudobagrus fulvidraco* (Richardson). Journal of Applied Ichthyology 25: 771-775.

Jenkins, R. L., Wilson, E. M., Angus, R. A., Howell, W. M., Kirk, M., Moore, R., Nance, M. and Brown, A. (2004). Production of androgens by microbial transformation of progesterone *in vitro*: A model for androgen production in rivers. Environmental Health Perspectives 112: 1508-11.

Jensen, K. M., Kahl, M. D., Makynen, E. A., Korte, J. J., Leino, R. L., Butterworth, B. C. and Ankley, G. T. (2004). Characterization of responses to the antiandrogen flutamide in a short-term reproduction assay with the fathead minnow. Aquatic Toxicology 70: 99-110.

Jiao, B. W. and Cheng, C. H. K. (2010). Disrupting actions of bisphenol A and malachite green on growth hormone receptor gene expression and signal transduction in seabream. Fish Physiology and Biochemistry 36: 251-261.

Jin, Y., Chen, R., Sun, L., Wang, W., Zhou, L., Liu, W. and Fu, Z. (2009). Enantioselective induction of estrogen-responsive gene expression by permethrin enantiomers in embryo-larval zebrafish. Chemosphere 74: 1238-1244.

Jobling, Nolan, M., Tyler, C. R., Brighty, G. and Sumpter, J. P. (1998). Widespread sexual disruption in wild fish. Environmental Science and Technology 32: 2498-2506.

Jobling, Burn, R. W., Thorpe, K., Williams, R. and Tyler, C. (2009a). Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute

to widespread sexual disruption in fish living in English rivers. Environmental Health Perspectives 117: 797-802.

Jobling, S. and Sumpter, J. P. (1993). Detergent components in sewage effluent are weakly oestrogenic to fish - an *in vitro* study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquatic Toxicology 27: 361-372.

Jobling, S., Reynolds, T., White, R., Parker, M. G. and Sumpter, J. (1995a). A variety of environmentally persistent chemicals, including some phthalate plasticizers are weakly estrogenic. Environmental Health Perspectives 103: 582-587.

Jobling, S., Reynolds, T., White, R., Parker, M. G. and Sumpter, J. P. (1995b). A variety of environmentally persistent chemicals, including some phthalate plasticisers, are weakly estrogenic. Environmental Health Perspectives 103: 582-587.

Jobling, S., Burn, R. W., Thorpe, K., Williams, R. and Tyler, C. (2009b). Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in English rivers. Environmental Health Perspectives 117: 797-802.

Jobling, S., Burn, R. W., Thorpe, K., Williams, R. and Tyler, C. (2009c). Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in english rivers. Environmental Health Perspectives 117: 797-802.

Jordan, J., Zare, A., Jackson, L. J., Habibi, H. R. and Weljie, A. M. (2012). Environmental contaminant mixtures at ambient concentrations invoke a metabolic stress response in goldfish not predicted from exposure to individual compounds alone. Journal of Proteome Research 11: 1133-1143.

Kang, I. J., Yokota, H., Oshima, Y., Tsuruda, Y. and Hano, T. (2003). Effects of 4-nonylphenol on reproduction of Japanese medaka, *Oryzias latipes*. Environmental Toxicology and Chemistry 22: 2438-2445.

Kang, I. J., Hano, T., Oshima, Y., Yokota, H., Tsuruda, Y., Shimasaki, Y. and Honjo, T. (2006). Anti-androgen flutamide affects gonadal development and reproduction in medaka (*Oryzias latipes*). Marine Environmental Research 62: S253-S257.

Kang, J. C., Jee, J. H., Koo, J. G., Keum, Y. H., Jo, S. G. and Park, K. H. (2010). Anti-oxidative status and hepatic enzymes following acute administration of diethyl phthalate in olive flounder *Paralichthys olivaceus*, a marine culture fish. Ecotoxicology and Environmental Safety 73: 1449-1455.

Kassim, N. M., McDonald, S. W., Reid, O., Bennett, N. K., Gilmore, D. P. and Payne, A. P. (1997). The effects of pre- and postnatal exposure to the nonsteroidal antiandrogen flutamide on testis descent and morphology in the albino swiss rat. Journal of Anatomy 190: 577-588.

Katsiadaki, I. (2207) The use of the stickleback as a sentinel and model species on ecotoxicology. In: Biology of the three-spined stickleback Eds. Ostlund-Nilsson, S., Mayer, I. and Huntingford, F. A. Marine Biology Series: 319-351. CRC Press; Boca Raton.

Katsiadaki, I., Morris, S., Squires, C., Hurst, M. R., James, J. D. and Scott, A. P. (2006). Use of the three-spined stickleback (*Gasterosteus aculeatus*) as a sensitive in vivo test for detection of environmental antiandrogens. Environmental Health Perspectives 114: 115-121.

Kavanagh, R. J., Balch, G. C., Kiparissis, Y., Niimi, A. J., Sherry, J., Tinson, C. and Metcalfe, C. D. (2004). Endocrine disruption and altered gonadal development in white perch (*Morone americana*) from the lower great lakes region. Environmental Health Perspectives 112: 898-902.

Kazeto, Y., Tosaka, R., Matsubara, H., Ijiri, S. and Adachi, S. (2011). Ovarian steroidogenesis and the role of sex steroid hormones on ovarian growth and maturation of the japanese eel. Journal of Steroid Biochemistry and Molecular Biology 127: 149-154.

Kefi, A. S., Kang'ombe, J., Kassam, D. and Katongo, C. (2012). Growth, reproduction and sex ratios in oreochromis andersonii (Castelnau, 1861) fed with varying levels of 17α-methyl testosterone. Journal of Aquaculture Research and Development 3: 155-155.

Kidd, K. A., Blanchfield, P. J., Mills, K. H., Palace, V. P., Evans, R. E., Lazorchak, J. M. and Flick, R. W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences of the United States of America 104: 8897-8901.

Kim, E. J., Kim, J. W. and Lee, S. K. (2002). Inhibition of oocyte development in japanese medaka (*Oryzias latipes*) exposed to di-2-ethylhexyl phthalate. Environment International 28: 359-365.

Kim, J. H., Lee, J. H. and Kim, S. Y. (2014a). Investigation of the source of residual phthalate in sundried salt. Journal of Food Protection 77: 480-484.

Kim, M., Song, N. R., Choi, J.-H., Lee, J. and Pyo, H. (2014b). Simultaneous analysis of urinary phthalate metabolites of residents in Korea using isotope dilution gas

chromatography-mass spectrometry. Science of the Total Environment 470: 1408-1413.

Kim, W.-S., Do, A., Yeh, D. and Cunningham, J. (2014c). Extraction of bisphenol-A and 17 beta-estradiol from water samples via solid-phase extraction (SPE). Reviews in Analytical Chemistry 33: 59-77.

Kinnberg, K. and Toft, G. (2003). Effects of estrogenic and antiandrogenic compounds on the testis structure of the adult guppy (*Poecilia reticulata*). Ecotoxicology and Environmental Safety 54: 16-24.

Kiparissis, Y., Metcalfe, T. L., Balch, G. C. and Metcalf, C. D. (2003). Effects of the antiandrogens, vinclozolin and cyproterone acetate on gonadal development in the Japanese medaka (*Oryzias latipes*). Aquatic Toxicology 63: 391-403.

Kloas, W., Lutz, I. and Einspanier, R. (1999). Amphibians as a model to study endocrine disruptors: Estrogenic activity of environmental chemicals *in vitro* and *in vivo*. The Science of the Total Environment 225: 59-68.

Knapczyk-Stwora, K., Durlej-Grzesiak, M., Ciereszko, R. E., Koziorowski, M. and Slomczynska, M. (2013). Antiandrogen flutamide affects folliculogenesis during fetal development in pigs. Reproduction 145: 265-276.

Koch, H. M., Drexler, H. and Angerer, J. (2003). An estimation of the daily intake of di(2-ethylhexyl)phthalate (DEHP) and other phthalates in the general population. International Journal of Hygiene Environmental Health 206: 77-83.

Koenen, S. and Cavas, T. (2008). Genotoxicity testing of the herbicide trifluralin and its commercial formulation treflan using the piscine micronucleus test. Environmental and Molecular Mutagenesis 49: 434-438.

Kogila Vani, A., Mohd Harun, A., Mazlin, M., Joseph, C. G. and Mahyar, S. (2013). Characterization of aromatic hydrocarbons in tropical coastal water of Sabah, Borneo. Asian Journal of Chemistry 25(7): 3773-3780.

Kolodziej, E. P., Gray, J. L. and Sedlak, D. L. (2003). Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. Environmental Toxicology and Chemistry 22: 2622-2629.

Kopera, I., Durlej, M., Hejmej, A., Knapczyk-Stwora, K., Duda, M., Slomczynska, M. and Bilinska, B. (2011). Differential expression of connexin 43 in adult pig testes during normal spermatogenic cycle and after flutamide treatment. Reproduction in Domestic Animals 46: 1050-1060.

Kopera, I., Durlej, M., Hejmej, A., Knapczyk-Stwora, K., Duda, M., Slomczynska, M., Koziorowski, M. and Bilinska, B. (2010). Effects of pre- and post- natal exposure to flutamide on connexin 43 expression in testes and ovaries of prepubertal pigs. European Journal of Histochemistry 54: 67-73.

Korner, W., Bolz, U., Sussmuth, W., Hiller, G., Schuller, W., Hanf, V. and Hagenmaier, H. (2000). Input/output balance of estrogenic active compounds in a major municipal sewage plant in germany. Chemosphere 40: 1131-42.

Kortner, T. M., Rocha, E. and Arukwe, A. (2009a). Previtellogenic oocyte growth and transcriptional changes of steroidogenic enzyme genes in immature female atlantic cod (*Gadus morhua*) after exposure to the androgens 11-ketotestosterone and testosterone. Comparative Biochemistry and Physiology 152: 304-313.

Kortner, T. M., Rocha, E. and Arukwe, A. (2009b). Androgenic modulation of early growth of atlantic cod (*Gadus morhua*) previtellogenic oocytes and zona radiata-related genes. Journal of Toxicology and Environmental Health 72: 184-195.

Kotula-Balak, M., Hejmej, A., Kopera, I., Lydka, M. and Bilinska, B. (2012). Prenatal and neonatal exposure to flutamide affects function of leydig cells in adult boar. Domestic Animal Endocrinology 42: 142-154.

Kuiper, R. V., Vethaak, A. D., Canton, R. F., Anselmo, H., Dubbeldam, M., van den Brandhof, E. J., Leonards, P. E. G., Wester, P. W. and van den Berg, M. (2008). Toxicity of analytically cleaned pentabromodiphenyl ether after prolonged exposure in estuarine european flounder (*Platichthys flesus*), and partial life-cycle exposure in fresh water zebrafish (*Danio rerio*). Chemosphere 73: 195-202.

Kwak, H. I., Bae, M. O., Lee, M. H., Lee, Y. S., Lee, B. J., Kang, K. S., Chae, C. H., Sung, H. J., Shin, J. S., Kim, J. H., Mar, W. C., Sheen, Y. Y. and Cho, M. H. (2001). Effects of nonylphenol, bisphenol A, and their mixture on the viviparous swordtail fish (*Xiphophorus helleri*). Environmental Toxicology and Chemistry 20: 787-795.

Labrie, F. and Crawford, D. (1995). Anti-androgens in treatment of prostate-cancer. Lancet 346: 1030-1031.

Lague, E. and Tremblay, J. J. (2008). Antagonistic effects of testosterone and the endocrine disruptor mono-(2-ethylhexyl) phthalate on INSL3 transcription in leydig cells. Endocrinology 149: 4688-4694.

Lange, R., Hutchinson, T. H., Croudace, C. P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G. H. and Sumpter, F. (2001). Effects of the synthetic estrogen 17a-

ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). Environmental Toxicology and Chemistry 20: 1216-1227.

Laurenson, J. P., Bloom, R. A., Page, S. and Sadrieh, N. (2014). Ethinyl estradiol and other human pharmaceutical estrogens in the aquatic environment: A review of recent risk assessment data. AAPS Journal 16: 299-310.

Leanos-Castaneda, O., Van Der Kraak, G., Lister, A., Sima-Alvarez, R. and Gold-Bouchot, G. (2002). O,p '-ddt induction of vitellogenesis and its inhibition by tamoxifen in nile tilapia (*Oreochromis niloticus*). Marine Environmental Research 54: 703-707.

Leaños-Castañeda, O. and Van Der Kraak, G. (2007). Functional characterization of estrogen receptor subtypes, eralpha and erbeta, mediating vitellogenin production in the liver of rainbow trout. Toxicology and Applied Pharmacology 224: 116-125.

Leblanc, G. A. (1980). Acute toxicity of priority pollutants to water flea (*Daphnia magna*). Bulletin of Environmental Contamination and Toxicology 24: 684-691.

Lee, S. T., Kime, D. E., Chao, T. M., Lim, H. S., Chou, R., Lam, T. J. and Tan, C. H. (1995) In vitro metabolism of testosterone by gonads of the grouper (*Epinephelus tauvina*) before and after sex inversion with 17 alpha-methyltestosterone. General and Comparative Endocrinology 99(1): 41-49.

Legler, J., Dennekamp, M., Vethaak, A. D., Brouwer, A., Koeman, J. H., van der Burg, B. and Murk, A. J. (2002). Detection of estrogenic activity in sediment-associated compounds using *in vitro* reporter gene assays. Science of the Total Environment 293: 69-83.

Leon, A., Teh, S. J., Hall, L. C. and Teh, F. C. (2007). Androgen disruption of early development in qurt strain medaka (*Oryzias latipes*). Aquatic Toxicology 82: 195-203.

Leonelli, C., Garcia, P. C. and Pereira, O. C. M. (2011). Copulatory efficiency and fertility in male rats exposed perinatally to flutamide. Reproductive Toxicology 31: 10-16.

Leusch, F. D., Chapman, H. F., Korner, W., Gooneratne, S. R. and Tremblay, L. A. (2005). Efficacy of an advanced sewage treatment plant in southeast Queensland, Australia, to remove estrogenic chemicals. Environment Science and Technology 39: 5781-6.

Liu, X., Li, D., Li, J., Rose, G. and Marriott, P. J. (2013). Organophosphorus pesticide and ester analysis by using comprehensive two-dimensional gas chromatography with flame photometric detection. Journal of Hazardous Materials 263: 761-767.

Loraine, G. A. and Pettigrove, M. E. (2006). Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed

wastewater in southern California. Environmental Science and Technology 40: 687-695.

Luellen, D. R., Vadas, G. G. and Unger, M. A. (2006). Kepone in James river fish: 1976-2002. Science of the Total Environment 358: 286-297.

Ma, D., Chen, L. and Lui, R. (2013). Decrease of antiandrogenic activity in gray water and domestic wastewater treated by the mbr process. Environmental Science-Processes and Impacts 15: 668-676.

Mahood, I. K., Hallmark, N., McKinnell, C., Walker, M., Fisher, J. S. and Sharpe, R. M. (2005). Abnormal leydig cell aggregation in the fetal testis of rats exposed to di (n-butyl) phthalate and its possible role in testicular dysgenesis. Endocrinology 146: 613-623.

Main, K. M., Mortensen, G. K., Kaleva, M. M., Boisen, K. A., Damgaard, I. N., Chellakooty, M., Schmidt, I. M., Suomi, A. M., Virtanen, H. E., Petersen, D. V., Andersson, A. M., Toppari, J. and Skakkebaek, N. E. (2006). Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. Environment Health Perspectives 114: 270-6.

Makynen, E. A., Kahl, M. D., Jensen, K. M., Tietge, J. E., Wells, K. L., Van Der Kraak, G. and Ankley, G. T. (2000a). Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). Aquatic Toxicology 48: 461-475.

Makynen, E. A., Kahl, M. D., Jensen, K. M., Tietge, J. E., Wells, K. L., Van der Kraak, G. and Ankley, G. T. (2000b). Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). Aquatic Toxicology 48: 461-475.

Manikkam, M., Tracey, R., Guerrero-Bosagna, C. and Skinner, M. K. (2013). Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. Plos One 8.

Mansour, A., Daba, A., Baddour, N., El-Saadani, M. and Aleem, E. (2012). Schizophyllan inhibits the development of mammary and hepatic carcinomas induced by 7,12 dimethylbenz(alpha)anthracene and decreases cell proliferation: Comparison with tamoxifen. Journal of Cancer Research and Clinical Oncology 138: 1579-1596.

Maradonna, F., Evangelisti, M., Gioacchini, G., Migliarini, B., Olivotto, I. and Carnevali, O. (2013). Assay of vtg, ers and ppars as endpoint for the rapid *in vitro* screening of the harmful effect of di-(2-ethylhexyl)-phthalate (DEHP) and phthalic acid (PA) in

zebrafish primary hepatocyte cultures. Toxicology *in vitro*: an international journal published in association with BIBRA 27: 84-91.

Marjani, M., Jamili, S. and Mostafavi, P. G. (2009). Influence of 17-alpha methyl testosterone on masculinization and growth in tilapia (*Oreochromis mossambicus*). Journal of Fisheries and Aquatic Science 4: 71-74.

Martinez, C., Ramirez, N., Gomez, V., Pocurull, E. and Borrull, F. (2013). Simultaneous determination of 76 micropollutants in water samples by headspace solid phase microextraction and gas chromatography-mass spectrometry. Talanta 116: 937-945.

Martyniuk, C. J., Spade, D. J., Blum, J. L., Kroll, K. J. and Denslow, N. D. (2011). Methoxychlor affects multiple hormone signaling pathways in the largemouth bass (*Micropterus salmoides*) liver. Aquatic Toxicology 101: 483-492.

Maschio, L. R., Cordeiro, R. S., Taboga, S. R. and Goes, R. M. (2010). Short-term antiandrogen flutamide treatment causes structural alterations in somatic cells associated with premature detachment of spermatids in the testis of pubertal and adult guinea pigs. Reproduction in Domestic Animals 45: 516-524.

Matsubara, M., Lokman, P. M., Senaha, A., Kazeto, Y., Ijiri, S., Kambegawa, A., Hirai, T., Young, G., Todo, T., Adachi, S. and Yamauchi, K. (2003). Synthesis and possible function of 11-ketotestosterone during oogenesis in eel (*Anguilla spp.*). Fish Physiology and Biochemistry 28: 353-354.

Matsusue, K., Peters, J. M. and Gonzalez, F. J. (2004). Pparbeta/delta potentiates ppargamma-stimulated adipocyte differentiation. FASEB Journal 18(2): 1477-1479.

Matta, M. B., Cairncross, C. and Kocan, R. M. (1998). Possible effects of polychlorinated biphenyls on sex determination in rainbow trout. Environmental Toxicology and Chemistry 17: 26-29.

Matta, M. B., Linse, J., Cairncross, C., Francendese, L. and Kocan, R. M. (2001). Reproductive and transgenerational effects of methylmercury or aroclor 1268 on *Fundulus heteroclitus*. Environmental Toxicology and Chemistry 20: 327-335.

Matthews, J., Celius, T., Halgren, R. and Zacharewski, T. (2000). Differential estrogen receptor binding of estrogenic substances: A species comparison. Journal of Steroid Biochemistry and Molecular Biology 74: 223-234.

McKinnell, C., Sharpe, R. M., Mahood, K., Hallmark, N., Scott, H., Ivell, R., Staub, C., Jegou, B., Haag, F., Koch-Nolte, F. and Hartung, S. (2005). Expression of insulin-like factor 3 protein in the rat testis during fetal and postnatal development and in relation

to cryptorchidism induced by in utero exposure to di (n-butyl) phthalate. Endocrinology 146: 4536-4544.

Mendis-Handagama, S. and Ariyaratne, H. B. S. (2001). Differentiation of the adult leydig cell population in the postnatal testis. Biology of Reproduction 65: 660-671.

Metcalfe, C. D., Metcalfe, T. L., Kiparissis, Y., Koenig, B. G., Khan, C., Hughes, R. J., Croley, T. R., March, R. E. and Potter, T. (2001). Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by *in vivo* assays with japanese medaka (*Oryzias latipes*). Environmental Toxicology and Chemistry 20: 297-308.

Mikkila, T. F. M., Toppari, J. and Paranko, J. (2006). Effects of neonatal exposure to 4-tert-octylphenol, diethylstilbestrol, and flutamide on steroidogenesis in infantile rat testis. Toxicological Sciences 91: 456-466.

Mitchell, R. T., Childs, A. J., Anderson, R. A., van den Driesche, S., Saunders, P. T. K., McKinnell, C., Wallace, W. H. B., Kelnar, C. J. H. and Sharpe, R. M. (2012). Do phthalates affect steroidogenesis by the human fetal testis? Exposure of human fetal testis xenografts to di-n-butyl phthalate. Journal of Clinical Endocrinology and Metabolism 97: E341-E348.

Miyamoto, H., Yeh, S. Y., Wilding, G. and Chang, C. S. (1998). Promotion of agonist activity of antiandrogens by the androgen receptor coactivator, ara70, in human prostate cancer DU145 cells. Proceedings of the National Academy of Sciences of the United States of America 95: 7379-7384.

Mohnke, M. and Buijten, J. (1993). Trace analysis of volatile halogenated hydrocarbons in water. Chromatographia 37: 51-56.

Montajami, S. (2012). Assessment the impact of 17 alpha -methyl testosterone on the growth and survival rate of golden barb fish, *Puntius gelius* (Hamilton, 1822). American-Eurasian Journal of Agricultural & Environmental Sciences 12: 1052-1055.

Montgomery, T. M., Brown, A. C., Gendelman, H. K., Ota, M. and Clotfelter, E. D. (2014). Exposure to 17 alpha-ethinylestradiol decreases motility and ATP in sperm of male fighting fish *Betta splendens*. Environmental Toxicology 29: 243-252.

Moody, S., Goh, H., Bielanowicz, A., Rippon, P., Loveland, K. L. and Itman, C. (2013). Prepubertal mouse testis growth and maturation and androgen production are acutely sensitive to di-n-butyl phthalate. Endocrinology 154: 3460-3475.

Moyer, B. and Nixon, M. L. (2012). Reproductive effects in f1 adult females exposed in utero to moderate to high doses of mono-2-ethylhexylphthalate (MEHP). Reproductive Toxicology 34: 43-50.

Muczynski, V., Lecureuil, C., Messiaen, S., Guerquin, M.-J., N'Tumba-Byn, T., Moison, D., Hodroj, W., Benjelloun, H., Baijer, J., Livera, G., Frydman, R., Benachi, A., Habert, R. and Rouiller-Fabre, V. (2012). Cellular and molecular effect of MEHP involving lxr alpha in human fetal testis and ovary. Plos One 7.

Mylchreest, E., Sar, M., Cattley, R. C. and Foster, P. M. D. (1999). Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. Toxicology and Applied Pharmacology 156: 81-95.

Naderi, M., Wong, M. Y. L. and Gholami, F. (2014). Developmental exposure of zebrafish (*Danio rerio*) to bisphenol-S impairs subsequent reproduction potential and hormonal balance in adults. Aquatic toxicology (Amsterdam, Netherlands) 148: 195-203.

FNakai, M., Tabira, Y., Asai, D., Yakabe, Y., Shimyozu, T., Noguchi, M., Takatsuki, M. and Shimohigashi, Y. (1999). Binding characteristics of dialkyl phthalates for the estrogen receptor. Biochemical and Biophysical Research Communications 254: 311-314.

Nakamura, A., Takanobu, H., Tamura, I., Yamamuro, M., Iguchi, T. and Tatarazako, N. (2013). Verification of responses of Japanese medaka (*Oryzias latipes*) to anti-androgens, vinclozolin and flutamide, in short-term assays. Journal of Apllied Toxicology

Nakari, T. (2004) Estrogenicity of municipal effluents assessed in vivo and in vitro. Environmental Toxicology, 19: 207-215.

Narita, K., Matsui, Y., Iwao, K., Kamata, M., Matsushita, T. and Shirasaki, N. (2014). Selecting pesticides for inclusion in drinking water quality guidelines on the basis of detection probability and ranking. Environment International 63: 114-120.

Nasu, M., Goto, M., Kato, H., Oshima, Y. and Tanaka, H. (2001). Study on endocrine disrupting chemicals in wastewater treatment plants. Water Science and Technology 43: 101-108.

Navas, J. M. and Segner, H. (2000). Antiestrogenicity of beta-naphthoflavone and PAHS in cultured rainbow trout hepatocytes: Evidence for a role of the aryl hydrocarbon receptor. Aquatic Toxicology 51: 79-92.

Nebot, C., Gibb, S. W. and Boyd, K. G. (2007). Quantification of human pharmaceuticals in water samples by high performance liquid chromatography-tandem mass spectrometry. Analytica Chimica Acta 598: 87-94.

Norman, A., Boerjeson, H., David, F., Tienpont, B., Gessbo, A. and Norrgren, L. (2006). Exposure of juvenile atlantic salmon (*Salmo salar*) to DEHP: Elimination and gonad differentiation. Marine Environmental Research 62: S270-S271.

Oehlmann, J., Schulte-Oehlmann, U., Tillmann, M. and Markert, B. (2000). Effects of endocrine disruptors on prosobranch snails (mollusca: Gastropoda) in the laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. Ecotoxicology 9: 383-397.

Ogunfowokan, A. O., Torto, N., Adenuga, A. A. and Okoh, E. K. (2006). Survey of levels of phthalate ester plasticizers in a sewage lagoon effluent and a receiving stream. Environmental Monitoring and Assessment 118: 457-480.

Oikari, A., Fragoso, N., Leppanen, H., Chan, T. and Hodson, P. V. (2002). Bioavailability to juvenile rainbow trout (*Oncorynchus mykiss*) of retene and other mixed-function oxygenase-active compounds from sediments. Environmental Toxicology and Chemistry 21: 121-128.

Olmstead, A. W. and LeBlanc, G. A. (2000). Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna*. Environmental Toxicology and Chemistry 19: 2107-2113.

Olujimi, O. O., Fatoki, O. S., Odendaal, J. P. and Daso, A. P. (2012). Chemical monitoring and temporal variation in levels of endocrine disrupting chemicals (priority phenols and phthalate esters) from selected wastewater treatment plant and freshwater systems in republic of South Africa. Microchemical Journal 101: 11-23.

Ortiz-Zarragoitia, M. and Cajaraville, M. P. (2005). Effects of selected xenoestrogens on liver peroxisomes, vitellogenin levels and spermatogenic cell proliferation in male zebrafish. Comparartive Biochemistry and Physiology 141: 133-144.

Ortiz-Zarragoitia, M., Trant, J. M. and Cajaraville, M. P. (2006). Effects of dibutyl phthalate and ethynylestradiol on liver peroxisomes, reproduction, and development of zebrafish (*Danio rerio*). Environmental Toxicology and Chemistry 25: 2394-2404.

Pan, G., Hanaoka, T., Yoshimura, M., Zhang, S., Wang, P., Tsukino, H., Inoue, K., Nakazawa, H., Tsugane, S. and Takahashi, K. (2006). Decreased serum free testosterone in workers exposed to high levels of di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP): A cross-sectional study in China. Environmental Health Perspectives 114: 1643-8.

Panter, G. H., Hutchinson, T. H., Lange, R., Lye, C. M., Sumpter, J. P., Zerulla, M. and Tyler, C. R. (2002). Utility of a juvenile fathead minnow screening assay for detecting (anti-)estrogenic substances. Environmental Toxicology & Chemistry 21: 319-326.

Parks, L. G., Lambright, C. S., Orlando, E. F., Guillette, L. J. Jr., Ankley, G. T. and Grey, L. E. Jr. (2001) Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. Toxicological Sciences 62(2): 257-67.

Patyna, P. J., Brown, R. P., Davi, R. A., Letinski, D. J., Thomas, P. E., Cooper, K. R. and Parkerton, T. F. (2006). Hazard evaluation of diisononyl phthalate and diisodecyl phthalate in a Japanese medaka multigenerational assay. Ecotoxicology and Environmental Safety 65: 36-47.

Pawlowski, S., van Aerle, R., Tyler, C. R. and Braunbeck, T. (2204) Effects of 17 alpha-ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay. Ecotoxicology and Environmental Safety 57(3): 330-345.

Perez, J. A., Downs, J. E. and Brown, P. J. (1976). Effects of dimethyl phthalate on growth of *Pseudomonas aeruginosa*. Bulletin of Environmental Contamination and Toxicology 16: 486-490.

Perobelli, J. E., Alves, T. R., de Toledo, F. C., Fernandez, C. D. B., Anselmo-Franci, J. A., Klinefelter, G. R. and Kempinas, W. D. G. (2012). Impairment on sperm quality and fertility of adult rats after antiandrogen exposure during prepuberty. Reproductive Toxicology 33: 308-315.

Pilla, D., Kavadi, A. K. M., Gurijala, P., Masuram, S., Delaney, M. S., Merchant, M. E. and Sneddon, J. (2009). Determination of selected chlorohydrocarbons and polyaromatic hydrocarbons by gas chromatography-mass spectrometry in soils in southwest louisiana. Microchemical Journal 91: 13-15.

Pollino, C. and Holdway, D. A. (2002). Reproductive potential of crimson-spotted rainbowfish (*Melanotaenia fluviatilis*) following short-term exposure to bass strait crude oil and dispersed crude oil. Environmental Toxicology 17: 138-145.

Pottinger, T. G., Katsiadaki, I., Jolly, C., Sanders, M., Mayer, I., Scott, A. P., Morris, S., Kortenkamp, A. and Scholze, M. (2013). Anti-androgens act jointly in suppressing spiggin concentrations in androgen-primed female three-spined sticklebacks - prediction of combined effects by concentration addition. Aquatic Toxicology 140: 145-156.

Purdom, C. E., Hardiman, P. A., Bye, V. J., Eno, N. C., Tyler, C. R. and Sumpter, J. P. (1994). Estrogenic effects of effluents from sewage treatment works. Chemistry and Ecology 8: 275-285.

Pusey, B. J., Arthington, A. H., Bird, J. R. and Close, P. G. (2001). Reproduction in three species of rainbowfish (Melanotaeniidae) from rainforest streams in northern Queensland, Australia. Ecology of Freshwater Fish 10: 75-87.

Qin, J., Chen, H., Cai, W., Yang, T. and Jia, X. (2011). Effect of di-n-butyl phthalate (DBP) on biochemistry indicators of *Thryssa hamiltonii*. Nanfang Shuichan Kexue 7: 29-34.

Qin, Z.-F., Qin, X.-F., Yang, L., Li, H.-T., Zhao, X.-R. and Xu, X.-B. (2007). Feminizing/demasculinizing effects of polychlorinated biphenyls on the secondary sexual development of *Xenopus laevis*. Aquatic Toxicology 84: 321-327.

Qing, Z., Dong, H., Zhu, B., Qu, J. and Nie, Y. (2013). A comparison of various rural wastewater treatment processes for the removal of endocrine-disrupting chemicals (EDCs). Chemosphere 92: 986-992.

Rajakumar, A., Singh, R., Chakrabarty, S., Murugananthkumar, R., Laldinsangi, C., Prathibha, Y., Sudhakumari, C. C., Dutta-Gupta, A. and Senthilkumaran, B. (2012). Endosulfan and flutamide impair testicular development in the juvenile asian catfish, *Clarias batrachus*. Aquatic Toxicology 110: 123-132.

Rajakumari, K., Sethuramalingam, T. A., Esakkiammal, B. and Ananth Kumar, Y. (2009). Influence of 17 alpha -methyl testosterone on growth performance and sex reversal of poecilia sphenops. Journal of Nature Conservation 21: 83-88.

Ramakrishnan, S. and Wayne, N. L. (2008). Impact of bisphenol-a on early embryonic development and reproductive maturation. Reproductive Toxicology 25: 177-183.

Rao, K., Li, N., Ma, M. and Wang, Z. (2014). *In vitro* agonistic and antagonistic endocrine disrupting effects of organic extracts from waste water of different treatment processes. Frontiers of Environmental Science and Engineering 8: 69-78.

Rawson, C. A., Lim, R. P. and Warne, M. St.J. (2008) Skeletal morphology and maturation of male *Gambusia holbrooki* exposed to sewage treatment plant effluent. Ecotoxicology and Environmental Safety 70(3): 453-461.

Rawn, D. F. K., Ryan, J. J., Sadler, A. R., Sun, W.-F., Weber, D., Laffey, P., Haines, D., Macey, K. and Van Oostdam, J. (2014). Brominated flame retardant concentrations in sera from the Canadian health measures survey (CHMS) from 2007 to 2009. Environment International 63: 26-34.

Reid, H. P. and Holdway, D. A. (1995). Early development of the australian crimson-spotted rainbowfish *Melanotaenia fluviatilis* (pisces: Melanotaeniidae). Marine and Freshwater Research 46: 475-480.

Richburg, J. H. and Boekelheide, K. (1996). Mono-(2-ethylhexyl) phthalate rapidly alters both sertoli cell vimentin filaments and germ cell apoptosis in young rat testes. Toxicology and Applied Pharmacology 137: 42-50.

Rodriguez-Gonzalez, N., Gonzalez-Castro, M. J., Beceiro-Gonzalez, E., Muniategui-Lorenzo, S. and Prada-Rodriguez, D. (2014). Determination of triazine herbicides in seaweeds: Development of a sample preparation method based on matrix solid phase dispersion and solid phase extraction clean-up. Talanta 121: 194-8.

Routledge, E. J., Parker, J., Odum, J., Ashby, J. and Sumpter, J. P. (1998). Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. Toxicology and Applied Pharmacology 153: 12-19.

Roy, V. K. and Krishna, A. (2013). The expression pattern of the glucose transporter glut-5 in the testis during the spermatogenic cycle of the vespertilionid bat scotophilus heathi. General and Comparative Endocrinology 191: 59-64.

Saaristo, M., Craft, J. A., Lehtonen, K. K., Bjork, H. and Lindstrom, K. (2009). Disruption of sexual selection in sand gobies (*Pomatoschistus minutus*) by 17 alpha-ethinyl estradiol, an endocrine disruptor. Hormones and Behavior 55: 530-537.

Sabo-Attwood, T., Kroll, K. J. and Denslow, N. D. (2004). Differential expression of largemouth bass (*Micropterus salmoides*) estrogen receptor isotypes alpha, beta, and gamma by estradiol. Molecular and Cellular Endocrinology 218: 107-118.

Salah, W. M., El-din-Azab, M. E., Hindi, A. D. and Shaheen, A. A. (2012). Some physiological studies on induced monosex nile tilapia using 17 alpha methyl testosterone. Proceedings of the 5th Global Fisheries and Aquaculture Research Conference, Faculty of Agriculture, Cairo University, Giza, Egypt, 1-3 October 2012: 209-224.

Sanchez-Bayo, F. and Hyne, R. V. (2014). Detection and analysis of neonicotinoids in river waters - development of a passive sampler for three commonly used insecticides. Chemosphere 99: 143-151.

Schettler, T. (2006). Human exposure to phthalates via consumer products. International Journal of Andrology 29: 134-139.

Scholz, N. (2003). Ecotoxicity and biodegradation of phthalate monoesters. Chemosphere 53: 921-926.

Schultz, I. R., Nagler, J. J., Swanson, P., Wunschel, D., Skillman, A. D., Burnett, V., Smith, D. and Barry, R. (2013). Toxicokinetic, toxicodynamic, and toxicoproteomic aspects of short-term exposure to trenbolone in female fish. Toxicological Sciences 136: 413-429.

Sebire, M., Allen, Y., Bersuder, P. and Katsiadaki, I. (2008). The model anti-androgen flutamide suppresses the expression of typical male stickleback reproductive behaviour. Aquatic Toxicology 90: 37-47.

Sebire, M., Katsiadaki, I., Taylor, N. G. H., Maack, G. and Tyler, C. R. (2011). Short-term exposure to a treated sewage effluent alters reproductive behaviour in the three-spined stickleback (*Gasterosteus aculeatus*). Aquatic Toxicology 105: 78-88.

Shanthanagouda, A. H., Patil, J. G. and Nugegoda, D. (2013). Effects of exposure to oestrogenic compounds on aromatase gene expression are gender dependent in the rainbowfish, *Melanotaenia fluviatilis*. Comparative Biochemistry and Physiology C-Toxicology and Pharmacology 157: 162-171.

Shen, O., Du, G., Sun, H., Wu, W., Jiang, Y., Song, L. and Wang, X. (2009). Comparison of *in vitro* hormone activities of selected phthalates using reporter gene assays. Toxicology Letters 191: 9-14.

Shi, W., Zhang, F.-X., Hu, G.-J., Hao, Y.-Q., Zhang, X.-W., Liu, H.-L., Wei, S., Wang, X.-R., Giesy, J. P. and Yu, H.-X. (2012). Thyroid hormone disrupting activities associated with phthalate esters in water sources from Yangtze river delta. Environment International 42: 117-23.

Shilling, A. D. and Williams, D. E. (2000). The non-aromatizable androgen, dihydrotestosterone, induces antiestrogenic responses in the rainbow trout. Journal of Steroid Biochemistry and Molecular Biology 74: 187-194.

Shioda, T. and Wakabayashi, M. (2000). Effect of certain chemicals on the reproduction of medaka (*Oryzias latipes*). Chemosphere 40: 239-243.

Siegel, R., Ward, E., Brawley, O. and Jemal, A. (2011). The impact of eliminating socioeconomic and racial disparities on premature cancer deaths. Cancer Journal for Clinicians 61: 212-236.

Simard, J., Luthy, I., Guay, J., Belanger, A. and Labrie, F. (1986). Characteristics of interaction of the antiandrogen flutamide with the androgen receptor in various target tissues. Molecular and Cellular Endocrinology 44: 261-270.

Singh, M. S. and Joy, K. P. (1998). Effects of administration of cyproterone acetate on seminal vesicle and testicular activity, and serum testosterone and estradiol-17-beta levels in the catfish *Clarias batrachus*. Acta Biologica Hungarica 49: 143-154.

Singh, R., Singh, A. K. and Tripathi, M. (2012). Effect of a non steroidal tamoxifen on the gonad and sex differentiation in nile tilapia, *Oreochromis niloticus*. Journal of Environmental Biology 33: 799-803.

Smeets, J. M. W., van Holsteijn, I., Giesy, J. P., Seinen, W. and van den Berg, M. (1999). Estrogenic potencies of several environmental pollutants, as determined by vitellogenin induction in a carp hepatocyte assay. Toxicological Sciences 50: 206-213.

Sohoni, P. and Sumpter, J. P. (1998). Several environmental oestrogens are also anti-androgens. Journal of Endocrinology 158: 327-339.

Sone, K., Hinago, M., Itamoto, M., Katsu, Y., Watanabe, H., Urushitani, H., Tooi, O., Guillette, L. J. Jr. and Iguchi, T. (2005) Effects of an androgenic growth promoter 17beta-trenbolone on masculinization of Mosquitofish (Gambusia affinis affinis).

Soto, A. M. and Sonnenschein, C. (1995). The e-screen assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. Environmental Health Perspectives 103: 113-123. General and Comparative Endocrinology 143(2): 151-160.

Soto, A. M., Calabro, J. M., Prechtl, N. V., Yau, A. Y., Orlando, E. F., Daxenberger, A., Kolok, A. S., Guillette, L. J., le Bizec, B., Lange, I. G. and Sonnenschein, C. (2004). Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in eastern nebraska, USA. Environmental Health Perspectives 112: 346-352.

Soverchia, L., Ruggeri, B., Palermo, F., Mosconi, G., Cardinaletti, G., Scortichini, G., Gatti, G. and Polzonetti-Magni, A. M. (2005). Modulation of vitellogenin synthesis through estrogen receptor beta-1 in goldfish (*Carassius auratus*) juveniles exposed to 17-b estradiol and nonylphenol. Toxicology and Applied Pharmacology 209: 236-243.

St-Louis, R., de Mora, S., Pelletier, M., Doidge, B., Leclair, D., Mikaelian, I. and Martineau, D. (2000). Hepatic butyltin concentrations in beluga whales (*Delphinapterus leucas*) from the St Lawrence estuary and northern Quebec, Canada. Applied Organometallic Chemistry 14: 218-226.

Staples, C. A., Peterson, D. R., Parkerton, T. F. and Adams, W. J. (1997a). The environmental fate of phthalate esters: A literature review. Chemosphere 35: 667-749.

Staples, C. A., Guinn, R., Kramarz, K. and Lampi, M. (2011). Assessing the chronic aquatic toxicity of phthalate ester plasticizers. Human and Ecological Risk Assessment 17: 1057-1076.

Staples, C. A., Adams, W. J., Parkerton, T. F., Gorschuch, J. W., Biddinger, G. R. and Reinert, K. H. (1997b). Aquatic toxicity of eighteen phthalate esters. Environmental Toxicology and Chemistry 16: 875-891.

Strauss, L., Santti, R., Saarinen, N., Streng, T., Joshi, S. and Makela, S. (1998). Dietary phytoestrogens and their role in hormonally dependent disease. Toxicology Letters 103: 349-354.

Stumpf, M., Ternes, T. A., Wilken, R. D., Rodrigues, S. V. and Baumann, W. (1999). Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil. Science of the Total Environment 225: 135-141.

Sudo, R., Tosaka, R., Ijiri, S., Adachi, S., Aoyama, J. and Tsukamoto, K. (2012). 11-ketotestosterone synchronously induces oocyte development and silvering-related changes in the japanese eel, *Anguilla japonica*. Zoological Science 29: 254-259.

Sugiyama, H., Murata, K., Iuchi, I., Nomura, K. and Yamagami, K. (1999). Formation of mature egg envelope subunit proteins from their precursors (choriogenins) in the fish, oryzias latipes: Loss of partial c-terminal sequences of the choriogenins. Journal of Biochemistry 125: 469-475.

Sumpter, J. P. (1995). Feminized responses in fish to environmental estrogens. Toxicology Letters 82/83: 737-742.

Sun, Y., Huang, H., Sun, Y., Wang, C., Shi, X. L., Hu, H. Y., Kameya, T. and Fujie, K. (2013). Ecological risk of estrogenic endocrine disrupting chemicals in sewage plant effluent and reclaimed water. Environmental Pollution 180: 339-344.

Sundt, R. C. and Bjorkblom, C. (2011). Effects of produced water on reproductive parameters in prespawning atlantic cod (*Gadus morhua*). Journal of Toxicology and Environmental Health 74: 543-554.

Tabata, A., Kashiwada, S., Ohnishi, Y., Ishikawa, H., Miyamoto, N. and Itoh, M. (2001). Estrogenic influences of estradiol-17b, *p*-nonylphenol and bis-phenol-A on Japanese medaka (*Oryzias latipes*) at detected environmental concentrations. Water Science and Technology 43: 109-116.

Tahara, R., Numabe, A. and Ishikawa, Y. (2014). Simultaneous determination of traces of herbicides in river water. Bunseki Kagaku 63: 127-132.

Takeuchi, S., Iida, M., Kobayashi, S., Jin, K., Matsuda, T. and Kojima, H. (2005a). Differential effects of phthalate esters on transcriptional activities via human estrogen receptors a and b, and androgen receptor. Toxicology 210: 223-233.

Takeuchi, S., Iida, M., Kobayashi, S., Jin, K., Matsuda, T. and Kojima, H. (2005b). Differential effects of phthalate esters on transcriptional activities via human estrogen receptors alpha and beta, and androgen receptor. Toxicology 210: 223-233.

Tan, B. L. L., Hawker, D. W., Muller, J. F., Leusch, F. D. L., Tremblay, L. A. and Chapman, H. F. (2007). Comprehensive study of endocrine disrupting compounds using grab and passive sampling at selected wastewater treatment plants in south-east Queensland, Australia. Environment International 33: 654-669.

Tan, G. H. (1995). Residue levels of phthalate-esters in water and sediment samples from the klang river basin. Bulletin of Environmental Contamination and Toxicology 54: 171-176.

Tashiro, Y., Takemura, A., Fujii, H., Takahira, K. and Nakanishi, Y. (2003). Livestock wastes as a source of estrogens and their effects on wildlife of manko tidal flat, okinawa. Marine Pollution Bulletin 47: 143-147.

Tellez-Banuelos, M. C., Ortiz, P. C., Jave, L. F., Siordia, V. H., Santerre, A. and Zaitseva, G. P. (2013). Effect of low concentration of endosulfan *in vivo* on cytotoxic activity of nonspecific cytotoxic cells and expression of granzyme gene in *Oreochromis niloticus* splenocytes. Fish and Shellfish Immunology 34: 1741-1742.

Thibaut, R. and Porte, C. (2004). Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. Journal of Steroid Biochemistry and Molecular Biology 92: 485-494.

Titley-O'Neal, C. P., Munkittrick, K. R. and MacDonald, B. A. (2011). The effects of organotin on female gastropods. Journal of Environmental Monitoring 13: 2360-2388.

Tokumoto, T., Tokumoto, M. and Nagahama, Y. (2005). Induction and inhibition of oocyte maturation by EDCs in zebrafish. Reproductive Biology and Endocrinology 3: 69.

Tomonari, Y., Kurata, Y., David, R. M., Gans, G., Kawasuso, T. and Katoh, M. (2006). Effect of di(2-ethylhexyl) phthalate (DEHP) on genital organs from juvenile common marmosets: I. Morphological and biochemical investigation in 65-week toxicity study. Journal of Toxicology and Environmental Health 69: 1651-1672.

Toomey, B. H., Monteverdi, G. H. and Di Giulio, R. T. (1999). Octylphenol induces vitellogenin production and cell death in fish hepatocytes. Environmental Toxicology and Chemistry 18: 734-739.

Trbovich, A. M., Sluss, P. M., Laurich, V. M., O'Neill, F. H., NacLaughlin, D. T., Donahoe, P. K. and Teixeira, J. (2001). Mullerian inhibiting substance lowers testosterone in luteinizing hormone-stimulated rodents. Proceedings of the National Academy of Sciences of the United States of America 98: 3393-3397.

Tricas, T. C., Maruska, K. P. and Rasmussen, L. E. L. (2000). Annual cycles of steroid hormone production, gonad development, and reproductive behavior in the Atlantic stingray. General and Comparative Endocrinology 118: 209-225.

Tyler, C. R., Jobling, S. and Sumpter, J. P. (1998). Endocrine disruption in wildlife - a critical review of the evidence. Critical Reviews in Toxicology 28: 319-361.

Tyler, C. R., and Routledge, E. J. (1998) Oestrogenic effects in fish in English rivers with evidence of their causation. Pure and Applied Chemistry 70: 319-361.

Uchida, M., Nakamura, H., Kagami, Y., Kusano, T. and Arizono, K. (2010). Estrogenic effects of o,p '- DDT exposure in japanese medaka (*Oryzias latipes*). Journal of Toxicological Sciences 35: 605-608.

Uren-Webster, T. M., Lewis, C., Filby, A. L., Paull, G. C. and Santos, E. M. (2010). Mechanisms of toxicity of di(2-ethylhexyl) phthalate on the reproductive health of male zebrafish. Aquatic Toxicology 99: 360-369.

Van den Belt, K., Berckmans, P., Vangenechten, C., Verheyen, R. and Witters, H. (2004). Comparative study on the in vitro in vivo estrogenic potencies of 17 beta-estradiol, estrone, 17 alpha-ethynylestradiol and nonylphenol. Aquatic Toxicology 66: 183-195.

van den Driesche, S., Scott, H. M., MacLeod, D. J., Fisken, M., Walker, M. and Sharpe, R. M. (2011). Relative importance of prenatal and postnatal androgen action in determining growth of the penis and anogenital distance in the rat before, during and after puberty. International Journal of Andrology 34: E578-E586.

Vazquez, G. R., Meijide, F. J., Da Cuna, R. H., Lo Nostro, F. L., Piazza, Y. G., Babay, P. A., Trudeau, V. L., Maggese, M. C. and Guerrero, G. A. (2009). Exposure to waterborne 4-tert-octylphenol induces vitellogenin synthesis and disrupts testis morphology in the south american freshwater fish Cichlasoma dimerus (Teleostei, Perciformes). Comparative Biochemistry and Physiology 150: 298-306.

Velasco-Santamaria, Y. M., Bjerregaard, P. and Korsgaard, B. (2013). Evidence of small modulation of ethinylestradiol induced effects by concurrent exposure to trenbolone in male eelpout (*Zoarces viviparus*). Environmental Pollution 178: 189-196.

Vethaak, A. D., Lahr, J., Schrap, S. M., Belfroid, A. C., Rijs, G. B. J., Gerritsen, A., de Boer, J., Bulder, A. S., Grinwis, G. C. M., Kuiper, R. V., Legler, J., Murk, T. A. J.,

Peijnenburg, W., Verhaar, H. J. M. and de Voogt, P. (2005). An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of the Netherlands. Chemosphere 59: 511-524.

Vigano, L., Arillo, A., Bottero, S., Massari, A. and Mandich, A. (2001). First observation of intersex cyprinids in the Po river (Italy). Science of Total Environment 269: 189-94.

Vo, T. T. B., Jung, E., Vu Hoang, D., Jung, K., Baek, J., Choi, K. and Jeung, E. (2009a). Differential effects of flutamide and di-(2-ethylhexyl) phthalate on male reproductive organs in a rat model. Journal of Reproduction and Development 55: 400-411.

Vo, T. T. B., Jung, E. M., Dang, V. H., Yoo, Y. M., Choi, K. C., Yu, F. H. and Jeung, E. B. (2009b). Di-(2 ethylhexyl) phthalate and flutamide alter gene expression in the testis of immature male rats. Reproductive Biology and Endocrinology 7: 104-

Vorkamp, K., Bossi, R., Bester, K., Bollmann, U. E. and Boutrup, S. (2014). New priority substances of the european water framework directive: Biocides, pesticides and brominated flame retardants in the aquatic environment of denmark. Science of the Total Environment 470: 459-468.

Wahbi, O. M. and Shalaby, S. H. (2010). Oral administration of testosterone in fish diet affect sex differentiation and testis development in tilapia. Research Journal of Agriculture and Biological Sciences 6: 946-952.

Wakeling, A. E., Furr, B. J. A., Glen, A. T. and Hughes, L. R. (1981). Receptor-binding and biological-activity of steroidal and non-steroidal antiandrogens. Journal of Steroid Biochemistry and Molecular Biology 15: 355-359.

Wakui, S., Shirai, M., Motohashi, M., Mutou, T., Oyama, N., Wempe, M. F., Takahashi, H., Inomata, T., Ikegami, M., Endou, H. and Asari, M. (2013). Effects of in utero exposure to di(n-butyl) phthalate for estrogen receptors alpha, beta, and androgen receptor of leydig cell on rats. Toxicology Pathology 42(5):877-887.

Walker, T. R. and MacAskill, D. (2014). Monitoring water quality in sydney harbour using blue mussels during remediation of the sydney tar ponds, Nova Scotia, Canada. Environmental Monitoring and Assessment 186: 1623-1638.

Wang, R., Wang, W., Ren, H. and Chae, J. (2014). Detection of copper ions in drinking water using the competitive adsorption of proteins. Biosensors and Bioelectronics 57: 179-85.

Wang, R., Liu, J., Yang, X., Lin, C., Huang, B., Jin, W. and Pan, X. (2013). Biological response of high-back crucian carp (*Carassius auratus*) during different life stages to

wastewater treatment plant effluent. Environmental Science and Pollution Research 20: 8612-8620.

Wang, Y. Q., Hu, W., Cao, Z. H., Fu, X. Q. and Zhu, T. (2005). Occurrence of endocrine-disrupting compounds in reclaimed water from Tianjin, China. Analytical and Bioanalytical Chemistry 383: 857-863.

Wei, Q., Nagi, R., Sadeghi, K., Feng, S., Yan, E., Ki, S. J., Caire, R., Tseng, D. and Ozcan, A. (2014). Detection and spatial mapping of mercury contamination in water samples using a smart-phone. Acs Nano 8: 1121-1129.

Weiss, J. M., Hamers, T., Thomas, K. V., van der Linden, S., Leonards, P. E. G. and Lamoree, M. H. (2009). Masking effect of anti-androgens on androgenic activity in european river sediment unveiled by effect-directed analysis. Analytical and Bioanalytical Chemistry 394: 1385-1397.

Werner, J., Ouellet, J. D., Cheng, C. S., Ju, Y. J. and Law, R. D. (2010). Pulp and paper mill effluents induce distinct gene expression changes linked to androgenic and estrogenic responses in the fathead minnow (*Pimephales promelas*). Environmental Toxicology and Chemistry 29: 430-439.

WHO (2012) State of the science of endocrine disrupting chemicals. Ed. Bergman A, Heindel J. J., Jobling S., Kidd K. A. and Zoeller R. T.

Williams, D. T. and Blanchfield, B. J. (1975). Retention, distribution, excretion, and metabolism of dibutyl phthalate-7-c-14 in rat. Journal of Agricultural and Food Chemistry 23: 854-858.

Williams, T. D., Caunter, J. E., Lillicrap, A. D., Hutchinson, T. H., Gillings, E. G. and Duffell, S. (2007). Evaluation of the reproductive effects of tamoxifen citrate in partial and full life-cycle studies using fathead minnows (*Pimephales promelas*). Environmental Toxicology and Chemistry 26: 695-707.

Wilson, W. B., Giam, C. S., Goodwin, T. E., Aldrich, A., Carpenter, V. and Hrung, Y. C. (1978). Toxicity of phthalates to marine dinoflagellate *Gymnodinium breve*. Bulletin of Environmental Contamination and Toxicology 20: 149-154.

Wine, R. N., Li, L. H., Barnes, L. H., Gulati, D. K. and Chapin, R. E. (1997). Reproductive toxicity of di-n-butyl phthalate in a continuous breeding protocol in sprague-dawley rats. Environmental Health Perspectives 105: 102-107.

Wong, C. I., Kelce, W. R., Sar, M. and Wilson, E. M. (1995). Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. Journal of Biological Chemistry 270: 19998-20003.

Wong, J. S. and Gill, S. S. (2002). Gene expression changes induced in mouse liver by di(2-ethylhexyl) phthalate. Toxicology and Applied Pharmacology 185: 180-196.

Woo, S., Denis, V. and Yum, S. (2014). Transcriptional changes caused by bisphenol A in *Oryzias javanicus*, a fish species highly adaptable to environmental salinity. Marine drugs 12: 983-98.

Woods, M. and Kumar, A. (2011). Vitellogenin induction by 17beta-ethynylestradiol in male murray rainbowfish (*Melanotaenia fluviatilis*). Environmental Toxicology and Chemistry 30: 2620-2627.

http://www.prostate.org.au/articleLive/ Accessed on April 10, 2014.

Xia, X., Crimmins, B. S., Hopke, P. K., Pagano, J. J., Milligan, M. S. and Holsen, T. M. (2009). Toxaphene analysis in great lakes fish: A comparison of GC-EI/Ms/Ms and gc-ecni-ms, individual congener standard and technical mixture for quantification of toxaphene. Analytical and Bioanalytical Chemistry 395: 457-463.

Xiaojing, L., Wenming, X., Hua, L., Liyang, Z., Shuiyuan, L., Xi, T., Minshi, C., Zhexiang, Z., Zhenyu, L., Bin, Q. and Guonan, C. (2013). Analysis of 16 phthalic acid esters in food simulants from plastic food contact materials by LC-ESI-MS/MS. Journal of Separation Science 36: 477-484.

Xu, H., Shao, X., Zhang, Z., Zou, Y., Wu, X. and Yang, L. (2013). Oxidative stress and immune related gene expression following exposure to di-n-butyl phthalate and diethyl phthalate in zebrafish embryos. Ecotoxicology and Environmental Safety 93: 39-44.

Xue, L., Zhang, D., Wang, T., Wang, X.-M. and Du, X. (2014). Dispersive liquid-liquid microextraction followed by high performance liquid chromatography for determination of phthalic esters in environmental water samples. Analytical Methods 6: 1121-1127.

Yan, H., Ye, C. M. and Yin, C. Q. (1995). Kinetics of phthalate ester biodegradation by *Chlorella pyrenoidosa*. Environmental Toxicology and Chemistry 14: 931-938.

Yao, P.-L., Lin, Y.-C. and Richburg, J. H. (2010). Mono-(2-ethylhexyl) phthalate-induced disruption of junctional complexes in the seminiferous epithelium of the rodent testis is mediated by MMP2. Biology of Reproduction 82: 516-527.

Ying, G. G. and Kookana, R. S. (2002a). Endocrine disruption: An australian perspective. Water 29: 42-45.

Ying, G. G., Kookana, R. S. and Ru, Y. J. (2002b). Occurrence and fate of hormone steroids in the environment. Environment International 28: 545-551.

Yu, L., Deng, J., Shi, X., Liu, C., Yu, K. and Zhou, B. (2010). Exposure to DE-71 alters thyroid hormone levels and gene transcription in the hypothalamic-pituitary-thyroid axis of zebrafish larvae. Aquatic Toxicology 97: 226-233.

Yun, S. H., Addink, R., McCabe, J. M., Ostaszewski, A., Mackenzie-Taylor, D., Taylor, A. B. and Kannan, K. (2008). Polybrominated diphenyl ethers and polybrominated biphenyls in sediment and floodplain soils of the saginaw river watershed, Michigan, USA. Archives of Environmental Contamination and Toxicology 55: 1-10.

Zacharewski, T. R., Meek, M. D., Clemons, J. H., Wu, Z. F., Fielden, M. R. and Matthews, J. B. (1998). Examination of the *in vitro* and *in vivo* estrogenic activities of eight commercial phthalate esters. Toxicological Sciences 46: 282-293.

Zanotelli, V. R. T., Neuhauss, S. C. F. and Ehrengruber, M. U. (2010). Long-term exposure to bis(2-ethylhexyl)phthalate (DEHP) inhibits growth of guppy fish (*Poecilia reticulata*). Journal of Applied Toxicology 30: 29-33.

Zhang, Z., Wang, Z., Li, Q., Zou, H. and Shi, Y. (2014a). Determination of trace heavy metals in environmental and biological samples by solution cathode glow discharge-atomic emission spectrometry and addition of ionic surfactants for improved sensitivity. Talanta 119: 613-619.

Zhang, Z., Ren, N., Kannan, K., Nan, J., Liu, L., Ma, W., Qi, H. and Li, Y. (2014b). Occurrence of endocrine-disrupting phenols and estrogens in water and sediment of the Songhua river, northeastern China. Archives of Environmental Contamination and Toxicology 66: 361-369.

Zhao, J.-L., Ying, G.-G., Yang, B., Liu, S., Zhou, L.-J., Chen, Z.-F. and Lai, H.-J. (2011). Screening of multiple hormonal activities in surface water and sediment from the pearl river system, south China, using effect-directed *in vitro* bioassays. Environmental Toxicology and Chemistry 30: 2208-2215.

Zhao, Z., Knowlton, K. F., Love, N. G. and Ogejo, J. A. (2010). Estrogen removal from dairy manure by pilot-scale treatment reactors. Transactions of the Asabe 53: 1295-1301.

Zheng, X., Zhang, B.-T. and Teng, Y. (2014). Distribution of phthalate acid esters in lakes of beijing and its relationship with anthropogenic activities. Science of Total Environment 476-477: 107-13.

Zhu, B., Lam, J. C. W., Yang, S. and Lam, P. K. S. (2013). Conventional and emerging halogenated flame retardants (HFRs) in sediment of Yangtze river delta (YRD) region, east China. Chemosphere 93: 555-560.

Zhu, L., Zhu, T., Ma, Y., Li, X. and Ni, Y. (2014). Determination of 17 kinds of phthalate esters in chicken flavor by dispersive solid phase extraction coupled with gas chromatography-mass spectrometry. Journal of Food Safety and Quality 5: 393-400.

Zia, A. I., Rahman, M. S. A., Mukhopadhyay, S. C., Pak-Lam, Y., Al-Bahadly, I. H., Gooneratne, C. P., Kosel, J. and Tai-Shan, L. (2013). Technique for rapid detection of phthalates in water and beverages. Journal of Food Engineering 116: 515-523.

CHAPTER 2

Effects of the commercial anti-androgen, flutamide on the biomarkers of reproduction in adult male Murray rainbowfish (*Melanotaenia fluviatilis*)

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CHAPTER 3

Effects of short-term exposure to the model anti-androgen, flutamide on reproductive function based endpoints in female Murray rainbowfish (*Melanotaenia fluviatilis*)

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CHAPTER 4

Effects of the model anti-androgen, flutamide on 17β-estradiol-induced hormonal imbalance in freshwater juvenile Murray rainbowfish (*Melanotaenia fluviatilis*)

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Effects of the model anti-androgen, flutamide on 17β-estradiol-induced hormonal imbalance in freshwater juvenile Murray rainbowfish (*Melanotaenia fluviatilis*)

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ABSTRACT

The aim of this study was to investigate if the reference anti-androgen, flutamide and 17β-estradiol work together to feminise juvenile Murray rainbowfish (*Melanotaenia fluviatilis*). Fish (60 days post hatch) were exposed to 25 ng/L of 17β-estradiol (E2), 25 µg/L of flutamide (Flu low), 250 µg/L of flutamide (Flu high), E2+Flu low and E2+Flu high in a semi-static set-up. After 35 days of exposure, concentrations of sex steroid hormones – 17β-estradiol and 11-keto testosterone (11-KT) were determined in the head tissue. The tail tissue was used to measure vitellogenin (VTG) concentration. The abdominal regions were used for histological investigation of the gonads. Co-treatment with Flu high and E2 resulted in significant reductions in weights and lengths in males and condition factor in females. Inter-sex was noted in Flu high and E2+Flu high treated fish. The development of spermatocytes in the testes was inhibited by E2 and this effect was accentuated after co-treatment with flutamide. Exposures to E2 resulted in precocious oocyte development in the ovaries which was further up-regulated when fish were co-exposed to E2 and flutamide. The E2 levels decreased significantly in the head of both males and females after co-exposures to flutamide and E2. Flutamide and E2 alone increased the 11-KT levels in both sexes. However, E2+Flu

low decreased 11-KT levels in males and increased them in females. Flutamide (low and high) induced VTG protein in the tails of both sexes. In males, VTG was induced in the tail tissue after exposure to flutamide but not E2. No significant increase of flutamide on E2-induced VTG concentration was noted. We conclude that anti-androgens do not add to the effects of estrogens due to different modes of action. However, they induce similar effects which can cause additive inhibition/stimulation of the gonad development.

Keywords: Flutamide, Estrogens, Sexual differentiation, Vitellogenin, Sex steroid hormones

1. Introduction

It has been well documented that certain industrial and agricultural chemicals and pharmaceuticals detected in fresh water worldwide have the potential to disrupt the endocrine system in fish. One of the earliest studies reported vitellogenin induction in male rainbow trout (*Oncorhynchus mykiss*) after exposure to the effluents from wastewater treatment plants (WWTPs) (Purdom *et al.,* 1994). Later, an intersex condition was detected in a roach (*Rutilus rutilus*) population living in water receiving discharges from sewage treatment plants (STPs) in the UK (Jobling *et al.,* 1998). With the development of sensitive analytical techniques, natural hormones like 17β-estradiol and synthetic hormones like ethinyl estradiol (EE2) were detected in minute amounts in environmental water samples and feminisation of wild fish was thought to be the result of exposures to estrogens (Desbrow *et al.,* 1998; Ternes *et al.,* 1999).

Generally, the endocrine disruption seen in wild fish is the result of simultaneous exposures to mixtures of chemicals with different modes of action (MoAs) (Hotchkiss *et al.,* 2008). It has been suggested that the environmental contaminants with different modes of

action (MoAs) can interact with each other resulting in cumulative estrogenic effects in wild-life (Kortenkamp and Altenburger 1998). Anti-androgens have been known to block the actions of endogenous androgens, thus, resulting in phenotypic feminisation "indicative of an estrogenic exposure" (Sohoni and Sumpter 1998). With the detection of anti-androgenic activity in freshwater (Ma *et al.,* 2013; Zhao *et al.,* 2011), it is being speculated that the endocrine disruption in fish has a "multicausal etiology" involving anti-androgens (Jobling *et al.,* 2009). Since, the causative agents remain uncertain and vary with location (Hotchkiss *et al.,* 2008), there is a need to understand and establish the effects of anti-androgenic chemicals, alone and in combination with estrogens, in robust laboratory experiments.

In this study, we investigated the effects of the "anti-androgen," flutamide (2-methyl-N-(4-nitro-3-[trifluoromethyl]phenyl)propanamide), alone and in combination with the natural estrogen, 17β-estradiol. Flutamide is a potent, non-steroidal reference anti-androgen that can competitively bind to the androgen receptors (ARs) and prevent the uptake of androgens, thus regulating androgen-mediated processes in the body. Flutamide is used therapeutically to treat prostate cancer in men and polycystic ovarian syndrome in women. It has been also been used as a model anti-androgen to study male reproductive development and toxicity testing in mammals. Flutamide administration during critical stages of development can interfere with the androgen-regulated processes and can cause irreversible changes in mammals (Mikkila *et al.,* 2006). For instance, *in utero* exposure to up to 30 mg/kg BW flutamide for 5 days resulted in the development of vaginal pouches in male pups (Goto *et al.,* 2004). Interestingly, feminisation of external genitalia and cryptorchidism were observed after *in utero* exposure to flutamide (Kassim *et al.,* 1997). However, these reproductive effects were not apparent when rats were administered flutamide during the postnatal period (Kassim *et al.,* 1997). The reproductive behaviour (like mounting a female)

was not affected after flutamide administration during the neonatal period. However, prenatal exposure continued until the neonatal phase lowered adult mounting behaviour (Brand and Slob 1991). Similarly, life-long exposure to 5 ng/L EE2 resulted in complete population failure with no fertilisation in zebrafish (*Danio rerio*). However, exposure to the same concentration for 40 days had no impact on reproduction in adult fish (Nash *et al.,* 2004).

Since mammals and fish share the similar vertebrate homology, it is hypothesised that the same endocrine disrupting chemicals (EDCs) that affect the sexual development in mammals during critical phases of growth can impair reproductive development in fish. Sexual differentiation during early stages of life cycle and a larger sensitive window of development in fish, suggest exposures to EDCs during juvenile stages have the potential to skew sex-ratios (van Aerle *et al.,* 2002). In one study, exposure of juvenile medaka (*Oryzias latipes*) to anti-androgens – vinclozolin (2500 µg/L) or cyperoterone acetate (10 µg/L) for 3 months induced inter-sex gonads and affected gametogenesis (Kiparissis *et al.,* 2003). Feminisation of juvenile fish after exposures to xenoestrogens like alkylphenols (octylphenol and *p*-nonylphenol) and pesticides (beta-hexachlorocyclohexane and methoxychlor) has been well documented (Gray and Metcalfe 1997; Gray *et al.,* 1999; Nimrod and Benson 1998; Wester *et al.,* 1985). However, similar studies on sexually undifferentiated fish using flutamide in combination with E2 are lacking. Although flutamide is not an environmental contaminant, it is used as the model AR-antagonist in toxicity testing since it binds competitively to the ARs (Ankley *et al.,* 2004) as opposed to other anti-androgens like vinclozolin and di-n-butyl phthalate which have multiple MoAs (Bhatia *et al.,* 2013; Bhatia *et al.,* 2014; Sohoni and Sumpter 1998).

Most of the laboratory studies using anti-androgens have been conducted on fish not native to and hence, not accustomed to Australian environment. Recently, we reported down-regulation in the hepatic expression levels of the genes encoding androgen receptors and hindrance in the transformation of spermatogonia to spermatozoa in Australian fish species – Murray rainbowfish (*Melanotaenia fluviatilis*) after exposure to 1000 µg/L flutamide for 7 days (Bhatia *et al.,* 2014). Adult Murray rainbowfish have earlier been used as the model species to assess the effects of EDCs via estrogen receptors (ERs) in the Australian riverine environment (Bhatia *et al.,* 2013; Bhatia *et al.,* 2014; Shanthanagouda *et al.,* 2013; Woods and Kumar 2011). The Murray rainbowfish (family Melanotaeniidae) is a freshwater fish species native to Australia. The fish reside in the Murray-Darling river system in New South Wales, Victoria and South Australia and spawn in the dry season (Pusey *et al.,* 2001). The embryonic development is completed in 7 – 9 days at 25 °C (Reid and Holdway 1995). The larvae are 3 – 5 mm at the time of hatching (Crowley *et al.,* 1986) and display sexual dimorphism in 6 – 8 months.

Based on the reported biologically active concentrations of flutamide in adult fish, we exposed juvenile Murray rainbowfish to 25 µg/L and 250 µg/L of flutamide, alone and in combination with 25 ng/L E2 for 35 days. The aim of the present study was to understand the effects of flutamide on E2-induced changes in gonadal development and the concentrations of sex steroid hormones and VTG in fish during their sensitive phase of development.

2. Materials and methods

2.1. Culture and maintenance of fish

Sexually immature, juvenile Murray rainbowfish (*Melanotaenia fluviatilis*) (60 days post hatch [dph]) were obtained from Aquarium Industries, Victoria. Fish were maintained in

artificial fresh water (fish loading 1 g/L) with pH 7 and > 90% dissolved oxygen (DO) and acclimatised for 2 weeks to the laboratory conditions. The temperature in the laboratory was maintained at 23 °C with 60 min gradual sunrise/sunset. Fish were fed 4% (w/w) body weight brine shrimp from the frozen stocks daily.

2.2. Chemicals

Flutamide (2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide) (CAS No. 13311-84-7) and 17β-estradiol (1,3,5(10)-estratrien-3,17β-diol) (CAS No. 50-28-2) were purchased from Sigma-Aldrich. Stock solutions (10 g/L) were prepared in methanol and stored at -20 °C in dark until use. Working solutions were prepared fresh daily. Fresh stock solutions were prepared every week.

2.3. Fish exposure

Fish were exposed in glass tanks in a semi-static set-up to 25 ng/L E2, 25 µg/L Flu (Flu low), 250 µg/L Flu (Flu high), E2+Flu low and E2+Flu high. Water control and 0.001% solvent control tanks were also included. All treatment and the solvent control tanks received the same concentration of methanol (0.001%). For each treatment (including the controls), there were four tanks, with three fish in each, for a total of twelve fish per treatment. The water in the tanks was renewed and spiked with fresh solutions daily. Fish were fed baby brine shrimp (4% w/w) daily at least 1 h before renewals. During the test, the temperature was maintained at 23 ± 1 °C with a 16 h:8 h light:dark photoperiod and a 60 min gradual sunrise/sunset. The temperature was measured every 30 sec using a temperature logger (Chen *et al.,*) placed in one of the testing tanks. The water quality parameters (pH, conductivity and DO) were measured every 24 h.

2.4. Sampling procedure

After 35 d of exposure to flutamide and/or E2 the fish were anaesthetised in 20 mg/L tricaine methanesulfonate (MS222). The lengths and weights of the fish were measured and condition factor (CF) calculated according to the formula, CF = body weight (g) / [total length (mm)]^3 x 100. The tail of the fish was cut at the anal fin, snap-frozen and stored at -80 °C for VTG analysis. The head was cut at the pectoral fin, snap-frozen and stored at -80 °C for the determination of the concentrations of sex steroid hormones. The abdominal regions were fixed in 10% formalin for histological investigation of the gonads. The use of head or tail homogenates for biochemical determination (such as VTG) has been recommended by the (OECD).

2.5. Chemical analyses

In order to measure the actual concentrations of E2 and flutamide during exposure, water samples (250 mL/tank) were collected in amber glass bottles from all the testing tanks on days 0, 1, 7, 14, 28 and 35 of the test. Water samples (1 L) were collected from the four replicates of each treatment for analysis. The samples collected before the start of the test represented day 0 samples. The water samples for days 1, 7, 14, 28 and 35 were collected 24 h after renewals and spiking of the fresh solutions. After collection, the water was spiked with 500 µL sulfuric acid to inhibit microbial growth. The water was filtered through glass filters with pore sizes 1.2 µm and 0.3 µm, respectively (Advantec) and loaded onto preconditioned cartridges (Oasis). The solid phase was eluted two times with methanol and dichloromethane (3 mL each) in glass tubes. The solvents were blown-down under a gentle stream of ultra-pure nitrogen gas at 30 °C.

The concentrations of E2 were measured on a gas chromatograph (GC) (Agilent) coupled with a triple quadrupole mass spectrometer (MS/MS). The residues were re-

dissolved in 900 µL pyridine and 10 µL N,O-bis(trimethylsilyl)-trifluoroacetamide (BFTSA) + trimethylchlorosilane (TMCS) (99:1). The vials were sealed and heated to 60 °C for 1 h. The GC inlet was lined with a 4 mm single tapered deactivated liner (Agilent) and used a splitless mode held at 250 °C. A 2 µL sample was injected onto the column. Analytes were separated on a 30 m x 0.25 mm x 0.25 mm MS column (Agilent) with 1 mL/min ultra-pure helium flow. The oven was at 280 °C with temperatures increasing at the rate of 5 °C/min. The GCMS-MS interface temperature was maintained at 250 °C. The electron impact (EI) voltage was set at 70 eV. Multiple reaction monitoring (MRM) was used to identify and quantify E2. The MRM transitions for E2 were were 416/326 and 416/285. One MRM value was used for quantification and the other for confirmation. All samples were run with a solvent delay of 8 min. The dwell times were 10 min for all MRMs to achieve 10 – 20 cycles across each peak for quantifications. The collision energies were 5 V.

For measurement of flutamide concentrations, the residues were dissolved in 1000 µL methanol. Liquid chromatography (LC) was performed on a Thermo-Finnigan Surveyor™ autosampler using 5 µL sample injected into an ODS Hypersil column (100 mm x 2.1 mm, particle size 3 µm). The temperatures of the column oven and autosampler were set at 30 °C and 10 °C, respectively. Separation was performed with a binary mobile phase at a flow rate of 250 mL/min. The first 2 .5 min of the flow of the chromatographic run was sent to waste via a 6-port-2-position valve installed post column in order to prevent the contamination of the ion source from matrix components. The optimised separation conditions were as follows: solvent A – acetonitrile and solvent B – a mixture of 0.1% formic acid and 10mM ammonium formate. The gradient elution was as follows: 95% solvent B (0 – 1 min) followed by an increase in the solvent A phase by 90% within 0.1 min. The solvent B was maintained at 90% for 6 min. The column was then re-equilibrated with 5% solvent A for 4 min and the total run

time was 10 min. Mass spectrometry (MS) was performed using Thermo TSQ Quadrupole mass spectrometer (ThermoFisher). The MS parameters were optimised as follows: spray voltage – 5 kV (ESI positive mode), sheath gas pressure – 40 arbitrary units, auxiliary gas pressure – 5 arbitrary units, capillary temperature – 350 °C and collision gas pressure – 1.5 mTorr. Ultra-pure nitrogen was used as a nebuliser gas and argon as collision gas. Optimisation of MS/MS parameters was performed by direct infusion of 100 µg/L flutamide at a flow rate of 10 µL/min. Collision energy (26/26) and tube lens voltages (73/73) were optimised for MRM transitions (275/186, 275/202). Data were acquired and processed using the Xcalibur 2.1 software.

Relative retention times of flutamide and E2 were monitored to ensure correct identification. The limit of quantification (LOQ) was set as the concentration of the chemical with a response area 20% of the lowest concentration of the standard. The limit of detection (LOD) was set as one-third of the LOQ.

2.6. Processing of tissues for histological investigation

The fish abdominal regions fixed in 10% formalin were processed for histological investigation by the method of (Bhatia et al., 2013). Briefly, the tissues were dehydrated in a graded series of ethanol and xylene in a tissue processor (Leica). The dehydrated tissues were embedded in paraffin using a paraffin-embedding centre (Leica). Rough trimming of the block containing the abdominal region of the fish was done on a rotary microtome (Microm International) till the gonadal cells began to appear. Serial vertical sections (4 – 5 µm) were then cut. The first section was taken from 50 µm depth. Five sections were cut and floated on a water bath at 40 °C for each fish. The sections were laid on a slide, dried and hydrated in a graded series of ethanol. The sections were stained with haematoxylin and eosin phloxine

(HD Scientific). The stained sections were photographed using an Olympus BX51 microscope and analysed with the software AnalySIS software (Olympus Soft Imaging Solutions GmbH 5.0 build 1235).

The histological investigation involved identification of the germ cells in the gonads. The germ cells in the ovaries were identified on the basis of their size and presence of cortical alveoli (Bhatia *et al.,* 2013) as follows: a) Perinucleolar oocytes – early primary growth oocytes with a large nucleus and homogenously staining dark cytoplasm. The follicular cells, vitellogenin granules and cortical alveoli were absent; b) Cortical alveolar oocytes – larger than the perinucleolar oocytes with a ring of cortical alveoli near the periphery; c) Early vitellogenic oocytes – larger than the cortical alveolar oocytes. The cytoplasm is filled with cortical alveoli and there is a beginning of vitellogenin accumulation; d) Late vitellogenic oocytes – most of the cytoplasm is filled with vitellogenin, cortical alveoli are pushed to the periphery of the oocyte. e) Mature oocytes – vitellogenesis is at its peak in these germ cells. These are the largest oocytes and yolk fills the cytoplasm; and f) Atretic oocytes – oocytes in any stage of development are atretic if they develop vacuolated nuclei, folding of oolemma, disorganisation of cytoplasm, hyperplasia or hypertrophy of follicular cells or impairment in yolk formation. The development of the ovaries was classified into the following stages: Stage 0 (immature) – This stage corresponded to immature ovary with only perinucleolar oocytes; Stage I (previtellogenic) – In addition to abundant previtellogenic oocytes, the cortical alveolar oocytes begin to appear; Stage II (early vitellogenic) – The ovaries in this stage were characterised by the appearance of early vitellogenic oocytes. Some cortical alveolar and perinucleolar oocytes were also present; Stage III (mature) – The mature ovaries had abundant late vitellogenic or spawning oocytes with accumulation of yolk vesicles; and

Stage IV (spent) – In this stage the germ cells in different stages had lost shape and turned atretic.

In the testes, the germ cells were categorised according to the method outlined by Bhatia and co-workers (Bhatia *et al.,* 2014) into: a) Spermatogonia – large cells with eosinophilic cytoplasm; found in clusters near the periphery of the testes; b) Spermatocytes – germ cells with moderate amounts of cytoplasm; c) Spermatids – smaller than the spermatocytes, with dense nucleus and minimal cytoplasm. The spermatogonia, spermatocytes and spermatids together constitute the germinal epithelium of the testes, representing immature germ cells; and d) Sperms – mature germ cells scattered in the tubular lumen. They are dark and dense cells with no apparent cytoplasm. The development of the testes was classified into the following stages: Stage 0 (immature): The testes were identified by the absence of spermatozoa in the tubular lumen. Abundant spermatocytes and spermatogonia were present. Sexually mature stages were further categorised into 3 stages: Stage I (early-spermatogenic): There was a beginning of the appearance of spermatozoa in this stage. Abundant spermatogonia and spermatocytes were present; Stage II (mid-spermatogenic): The mid spermatogenic stage was identified by the presence of approximately similar proportions of the spermatocytes, spermatids and spermatozoa along with moderately developed interstitial tissue; and Stage III (mature): Proliferation and development of germ cells was apparent. All types of germ cells were present. However, fish in this stage had abundant spermatozoa.

2.7. Vitellogenin analysis

A semi-quantitative enzyme linked immunosorbent assay (ELISA) method was used to measure the relative levels of VTG in the fish (Bhatia et al., 2013). The tails were thawed

on ice, weighed and homogenised in 1:10 (w/v) ice-cold buffer (pH 7.4) (100 mM KCl, 19 mM KH_2PO_4, 0.02 M HEPES, 1 mM EDTA and 1 mM dithioreitol) using 1 mm zirconium oxide beads (ThermoFisher Scientific) on Fastprep-24 (MP-Biomedicals) at 6 m/s for 1 min. The homogenate was centrifuged at 1500 *g* for 60 min at 4°C and the supernatant was diluted 10 times in phosphate buffer saline (PBS). The diluted homogenate (100 µL) was added to the white binding plates (Greiner) and incubated overnight at 4 °C. Serial dilutions (up to 248000 times) of plasma from female fish were run as positive controls. The wells were washed three times with wash buffer (0.05% Tween in PBS) and 200 µL of the blocking buffer (BB) was added. The plate was incubated at room temperature for 1 h after which the BB was discarded and 100 µL of the 1:2500 primary antibody (BALB/c mice anti-rainbowfish) (AusMAb) in BB was added to each well. The plate was again incubated at room temperature for 2 h. The wells were washed and 100 µL of the 2° antibody (1:4000 anti-mouse IgG conjugated to horseradish peroxidase) was added. After incubation for 2 h at room temperature, the wells were washed five times with the wash buffer and 100 µL of west Femto sensitivity substrate (ThermoFisher scientific) was added. The fluorescence was read at 405 nm using a luminometer (BMG Labtech). Protein concentrations were measured by the Bradford method. The relative results were reported as arbitrary units per mg protein.

2.8. Measurement of steroid hormone concentrations

Concentrations of E2 and 11-KT were measured in the head homogenates by ELISA using enzyme immunoassay (EIA) kits (Cayman Chemicals) (Mills *et al.,* 2010). The snap-frozen heads were thawed on ice, weighed and homogenised in 1:10 (w/v) homogenisation buffer (pH 7.4) (100 mM KCl, 19 mM KH_2PO_4, 0.02 M HEPES, 1 mM EDTA and 1 mM dithioreitol) using 1 mm zirconium oxide beads (ThermoFisher Scientific) on Fastprep-24 (MP-Biomedicals) at 6 m/s for 1 min. The homogenate was centrifuged at 1500 *g* for 60 min

and diluted two times in phosphate buffer saline (pH 7.4). The wells of the 96-well plate used were coated with anti-rabbit IgG antibody. E2 standards (6.6 pg/mL – 4000 pg/mL) or 11-KT standards (0.78 pg/mL – 100 pg/mL) were added to wells. Samples (50 µL), in triplicates, were added per well. 100 µL and 50 µL of EIA buffer were added to the wells designated as non-specific binding (NSB) and maximum binding (B_0), respectively. E2 (or 11-KT) acetyl cholinesterase (AChE) tracer (50 µL) was added to each well except to the total activity (TA) and the blank (Blk) wells. Then 50 µL E2 (or 11-KT) rabbit anti-serum were added to each well except to the TA, NSB and Blk wells and the plate was incubated at room temperature for 60 min for E2 measurement (or for 18 h at 4 °C for 11-KT measurement). After incubation, the wells were washed five times with wash buffer to remove unbound reagents. Ellman's reagent (200 µL) was added to each well. At this stage 5 µL of the E2 (or 11-KT) tracer was added to the TA wells and the plate was placed on an orbital shaker for 60 – 90 min for E2 measurement (or 90 – 120 min for 11-KT measurement). The luminescence was recorded at 405 nm using a luminometer (Thermo Labsystems multiskan ascent microplate photometer).The intensity of the yellow colour was inversely proportional to the amount of steroid in the sample.

The average absorbance reading of the NSB was subtracted from the average absorbance of B_0 to obtain the corrected B_0. The average NSB absorbance was subtracted from the average sample/standard absorbance and was divided by the corrected $B_0$to obtain B/B_0 (sample or standard bound/maximum bound). The $\%B/B_0$ for the standards was plotted against the standard concentration using linear (y) and log (x) axes and a four-parameter logistic fit was performed. The concentration of the hormones in the samples was determined by reading the value corresponding to $\%B/B_0$ on the x-axis.

2.9. Data analyses

All statistical analyses were performed on SigmaPlot 12.3. Normality of the data was confirmed using Shapiro-Wilk test. The data were subjected to one-way analysis of variance (ANOVA) to determine differences between treatment means. The Holm-Sidak test was used to determine significant differences between the groups. Differences in the proportions of the sexes and frequency of intersex condition between the controls and flutamide and/or E2 treatments was analysed by z-test. The significance was set at $p \leq 0.05$. Correlation analysis was carried and the Pearson correlation coefficient (r) was calculated for E2, 11-KT and VTG concentrations and the CFs.

3. RESULTS

3.1. Water chemistry of the exposure tanks

The measured concentrations of E2 and flutamide before the start of exposures were between 90 – 115% of the nominal concentrations. After 24 h of exposure, 60 – 80% of the nominal concentration of E2 was recovered from the testing tanks. More than 80% of the nominal concentration of flutamide was detected after 24 h of spiking. The LOD of the methods of determination of E2 and flutamide were 2 ng/L and 10 ng/L, respectively. The LOQs for E2 and flutamide were 6 ng/L and 30 ng/L, respectively. E2 was not detected in the flutamide treatment tanks and flutamide was not detected in E2 treatment tanks. Neither of the two test chemicals was detected in the water or solvent control tanks (Supplementary Table 1).

3.2. Water quality and physical conditions during fish exposure

During the entire duration of the exposure, the temperature was maintained at 23 ± 0.1 °C. The water quality parameters in the E2 and/or flutamide treatment tanks did not differ

significantly from those in the control tanks. The pH of the water across the treatments varied within the neutral range of 6.8 and 7.1. The DO levels in the water were above 80% in all tanks. Conductivity was maintained between 1185 and 1251 µS/cm (Supplementary Table 1).

3.3. Mortality and morpho-anatomical indices

No treatment-related mortality or abnormal behaviour was observed in fish after exposures to E2 and/or flutamide for 35 days. Weight of males significantly reduces after exposures to Flu low. Similar effects were seen in females after treatment with Flu high. Co-treatment with Flu high also reduced weights in males ($p \leq 0.05$) (Figures 1A, 1B). Exposure to E2 or Flu high alone resulted in significantly smaller females. However, in combination (E2 and Flu high) caused reduction in length in males ($p \leq 0.05$) (Figures 1C, 1D). Neither treatment had any significant effect on the CF in males. Flutamide (low or high) increased the CF in females. In combination with Flu high, E2 decreased the CF in females ($p \leq 0.05$) (Figures 1E, 1F).

3.4. Histological investigation of the gonads

The ovaries of the fish in controls as well as across all treatments contained tightly – packed perinucleolar, cortical alveolar and early vitellogenic oocytes suggesting normal development. In water and solvent controls, the fish contained abundant perinucleolar and cortical alveolar oocytes. A few early vitellogenic oocytes could be seen. The ovaries were in Stage I (Previtellogenic) of development (Figure 2A). The ovaries of the fish exposed to E2, Flu low, Flu high, E2+flu low and E2+flu high contained more of early vitellogenic oocytes and were in Stage II (Early vitellogenic) of development. Interstitial fibrosis was noted in the ovaries of the fish exposed to E2+flu high (Figures 2B, 2C, 2D). Qualitative assessment revealed no effect of flutamide on E2-induced precocious oocyte development.

Histological assessment of the testes of the control fish revealed the presence of spermatocytes, spermatids and spermatozoa in approximately similar proportions. The germ cells were compactly arranged and the testes were in the Stage II (mid-spermatogenic) (Figure 3A). Treatment with E2, alone an in combination with Flu low resulted in reduction in the proportion of spermatozoa in the tubular lumen. The testes of the fish in these treatments were in Stage I (early spermatogenic) (Figures 3B, 3C). An absence of spermatozoa was noted in fish treated with E2+flu high. The testes were in Stage 0 (immature) with abundant spermatocytes (Figure 3D). An increase in the height of germinal epithelium was noted in all treatments. Testes of the fish in E2 and E2+flu high treatments were vacuolated. In addition, interstitial fibrosis was noted in E2 treatment. Co-treatment with Flu high amplified the inhibitory effect of E2 on sperm development.

After 35 days of test, 50 – 56% fish were females in water and solvent controls. Incidences of intersex condition were seen in fish treated with E2, Flu high, E2+flu low and E2+flu high (Figure 4). Highest incidence (4 fish) of inter-sex gonads was found in Flu high treatment. Treatment with E2 did not affect the proportion of the females. Exposure to Flu low resulted in 70% females. This number declined to 67 after co-treatment with E2 (E2+flu low). Flu high resulted in 42% females which reduced to 33% when co-treated with E2 (E2+flu high). The 45 – 50% frequency of males in the controls declined to 36% after treatment with E2. The proportion further decreased 33% and 25% after exposures to Flu low and Flu high, respectively. A sharp decline to 17% male frequency was noted after exposure to E2+flu low. The changes in the sex-ratios and the frequency of inter-sex after treatment with flutamide and/or E2 were not significant (Supplementary Figure 1).

3.5. Hormone concentrations

Treatment with E2 did not result in significant changes in the E2 concentration in head tissue in males and females ($p \leq 0.05$). However, flutamide, alone and in combination with E2, significantly lowered the levels of E2 in both males as well as in females ($p \leq 0.05$) 5B). After 35 days of exposure to E2, a significant rise in 11-KT levels was measured in the head of both males and females ($p \leq 0.05$). In males, treatment with E2 or Flu low resulted in up to 80% increase in the 11-KT levels in comparison to the controls. However, their combined treatment (E2+flu low), decreased the 11-KT concentration in heads by up to 90%. A 37% increase in the 11-KT levels was noted in Flu high treated males. In females, E2+flu low caused an additive effect when administered in combination, resulting in a 70% increase in 11-KT levels. Interestingly, the three-time increase in the 11-KT levels noted in Flu high-treated females was brought at par to the controls when co-exposed with E2+flu high (Figures 5C, 5D). Decreases in E2/11-KT ratios in males (up to 93%) and in females (up to 88%) were noted after flutamide treatments (Figures 5E, 5F).

3.6. Vitellogenin concentrations

Vitellogenin protein was induced in the tails of the females but not in the males after exposure to E2. Flutamide (low and high) resulted in an increase in VTG in both males and females ($p \leq 0.05$). Co-treatment of E2 with Flu high caused the highest inductions (70 – 75%) in VTG levels. However, flutamide did not significantly amplify the E2-induced VTG levels in juvenile fish (Figures 6A, 6B).

There was no correlation between the concentrations of E2, 11-KT and VTG, the CFs and the proportions of the sexes. Treatment of the juvenile fish with E2 in combination with flutamide, did not accentuate the estrogenic effects.

4. DISCUSSION

The aim of the present study was to progress our understanding of the changes in gonadal development and the associated concentrations of sex steroid hormones and VTG caused by flutamide, alone and in combination with E2 in sexually undifferentiated, juvenile Murray rainbowfish. Histological analysis showed impairment in spermatogenesis after exposures to flutamide and the effect was more pronounced when treated with flutamide in combination with E2. In contrast, precocious development of the oocytes in the ovaries and induction in VTG concentration was noted in fish after E2+Flu high exposure. This study demonstrated that treatment with flutamide and/or E2 can cause feminising effects in juvenile Murray rainbowfish.

Reductions in the sizes of juvenile guppies were noted after exposures to three anti-androgens – the fungicide vinclozolin, the insecticide DDT and reference anti-androgen flutamide (Bayley *et al.,* 2002). This is in agreement with mammalian study that reported weight reductions in F2 generations after *in utero* exposure of rats exposed to another anti-androgen, di-n-butyl phthalate (DnBP) (Mylchreest *et al.,* 1999). There has been little research on the effects of anti-androgens on body growth. It has been demonstrated that EE2 and flutamide decrease the expression of the genes regulating the physiological processes like somatic growth [growth hormone (GH), growth hormone receptor (GHR), IGF-I, insulin-like growth factor-I receptor (IGF-I), thyroid hormone receptor α (THRα) and β (THRβ)] (Filby *et al.,* 2007). Several studies have reported decrease in plasma thyroxine (T3) concentrations after E2 exposures in Eurasian perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiss*) and eel (*Anguilla anguilla*) (Cyr and Eales 1990; Mandiki *et al.,* 2005; Olivereau *et al.,* 1981). Thyroxine has been reported to increase *in vivo* and *in vitro* levels of insulin growth

factor-I (IGF-I) mRNA, a somatic growth stimulator, in tilapia (Schmid *et al.,* 2003). In addition, IGF-I has been demonstrated to be involved in reproductive pathways like initiation of gonadotropin-release by pitutary cells in coho salmon (*Oncorhynchus kisutch*) and down-regulation of androgen production in the ovaries of red seabream (*Pagrus major*) (Baker *et al.,* 2000; Kagawa *et al.,* 2003). Interestingly in our recent studies, treatment of sexually mature male and female Murray rainbowfish with up to 1000 µg/L of flutamide for 7 days did not have any significant effects on body growth(Bhatia *et al.,* 2014). This could be due to different stage in life-cycle of the fish, concentration of flutamide used and the length of the exposure. Flutamide and E2 seem to target a regulatory process involved specifically in growth. Based on the data from the present study, we hypothesise that the growth-inhibitory effects of flutamide and/or E2 could be regulated in a gender-specific manner. We propose that exposure to flutamide and/or E2 during juvenile stage can adversely affect body growth, as assessed from the CF, in females. However, we did not investigate the changes in the expression levels of the genes involved in growth in the present study.

The present study confirmed the earlier reports that flutamide adversely affects normal testicular development. In general, histological evaluation showed that fish exposed to E2, Flu low and Flu high had fewer spermatozoa in the testicular tubule. The reduction in the proportion of the sperms was more evident when E2 was administered in combination with flutamide. Decrease in sperm count, testicular atrophy and/or spermatogonial proliferation after treatment with E2 have been demonstrated in juvenile flounder (*Paralichthys dentatus*), rainbow trout (*Salmo gairdneri*) and chub (*Leuciscus cephalus)* (Billard *et al.,* 1981; Flammarion *et al.,* 2000; Zaroogian *et al.,* 2001). Reductions in the sperm count and the number of spermatogenic cysts have been reported in guppy fed a diet containing up to 100 µg flutamide per mg fodder for 30 days (Baatrup and Junge 2001; Kinnberg and Toft 2003).

Treatment of juvenile Asian catfish with 33 µg/L flutamide for 50 days revealed slow progression of spermatogonia to spermatocytes (Rajakumar *et al.,* 2012). A 60% reduction in the number of mature sperms in juvenile guppy after treatment with anti-androgens – vinclozolin and DDE – suggested a decrease in male reproductive fitness (Bayley *et al.,* 2002). We have recently reported reduction in the proportion of spermatozoa after treatment with up to 1000 µg/L of flutamide or another mammalian anti-androgen, DnBP in sexually mature Murray rainbowfish (Bhatia *et al.,* 2014; Bhatia *et al.,* 2014). Dramatic reductions in sperm count have also been reported after short-term exposures to EE2 in fathead minnow, zebrafish, eelpout (Salierno and Kane 2009; Van den Belt *et al.,* 2002; Velasco-Santamaria *et al.,* 2010).

We reported reductions in the hepatic expressions of the genes for ARα and ARβ in male Murray rainbowfish after treatment with up to 1000 µg/L flutamide for 7 days (Bhatia *et al.,* 2014). Filby and co-workers have reported decrease in the expression of genes for ARα in the testes of male and the liver of female fathead minnow treated with 320 µg/L flutamide for 21 days (Filby *et al.,* 2007). Mammalian studies suggest the roles of ARs in the normal process of spermatogenesis. The seminiferous tubules of AR knock-out mice lacked mature spermatozoa, suggesting spermatogenic arrest at pachytene stage (Yeh *et al.,* 2002). Lack of AR expression in the Leydig cells also affects steroidogenesis in mice reviewed in (Wang *et al.,* 2009). In addition, the displacement of testosterone by flutamide from ARs (Ankley *et al.,* 2004; Makynen *et al.,* 2000; Simard *et al.,* 1986) could have adversely affected androgen-regulated processes. Any interference in the androgen-mediated process in the body can be communicated to the germ cells via autocrine or paracrine signalling to regulate spermatogenesis (Collins *et al.,* 2003). Recent studies using rainbow trout have confirmed the role of testosterone in up-regulating androgen-responsive genes in the somatic cells of the

testis (Rolland *et al.,* 2013). We believe that treatment with flutamide could have affected the androgen responsiveness in the Leydig or the Sertoli cells in the testes which arrested the development of spermatogonia into spermatozoa. However, this hypothesis needs validation. The effects of flutamide on germ cell development in testes were more pronounced after co-treatment with E2, which could be due to their similar MoAs at molecular level. Down-regulation of the expression of genes like anti-Mullerian hormone (*amh*), doublesex and mab-3 related transcription factor 1 (*dmrt1*) and/or Sry-related HMG box (*sox9*) involved in testicular differentiation has been reported in fish after treatment with flutamide (Filby *et al.,* 2007; Leon *et al.,* 2008; Rajakumar *et al.,* 2012). Similar findings have been reported after exposures to estrogens like E2 and EE2 in fish (Marchand *et al.,* 2000; Schulz *et al.,* 2007), turtles (Barske and Capel 2010) and mammals (Pask *et al.,* 2010). In addition, E2 treatment has been reported to decreases ARα gene expression in western mosquitofish (*Gambusia affinis*) (Huang *et al.,* 2012). The interstitial fibrosis observed in E2, E2+Flu low and E2+Flu high treatments is in consistency with other *in vitro* (Bouma *et al.,* 2003) and *in vivo* (Gimeno *et al.,* 1998; Karels *et al.,* 2001; Rasmussen and Korsgaard 2004) studies reporting testicular fibrosis after exposures to estrogens in fish.

Histological analysis of the gonads revealed precocious development of the ovaries in fish treated with E2 and/or flutamide. This is in consistency with an earlier study where treatment of 100 dph catfish (*Clarias batrachus*) to 33µg/L flutamide, alone or in combination with an estrogenic pesticide – endosulfan – for 50 days resulted in higher frequency of previtellogenic oocytes (Chakrabarty *et al.,* 2012). Induction in the transcripts for ERs after estrogenic exposure has been well documented. Some studies have reported similar results after treatment with flutamide in fish (Filby *et al.,* 2007), mammals (West *et al.,* 1990) and humans (KruithofDekker *et al.,* 1996). However, the mechanism by which

flutamide can upregulate ERs is not fully understood. An upregulation of ERs could have initiated activation of ER-regulated pathways of which VTG synthesis is of prime importance. We believe high concentration of VTG and its increased incorporation into the oocytes could have resulted in a higher proportion of vitellogenic oocytes after treatment with flutamide and/or E2.

It is likely that more 11-KT was produced in the brain (head tissue) to compensate for the demasulinisation after flutamide and/or E2 exposures. An increase in the androgen levels after treatment with anti-androgens like flutamide and vinclozolin has been reported in fathead minnow (Jensen *et al.,* 2004; Martinovic *et al.,* 2008). Flutamide has been demonstrated to increase the expression of the genes for enzymes involved in androgen biosynthesis in fish and rats (CYP11A1, CYP17, 3β-HSD) (Filby *et al.,* 2007; Ohsako *et al.,* 2003). Higher levels of circulating androgens after treatment with flutamide could also be due to their displacement from the ARs by flutamide (Ankley *et al.,* 2004). In addition, flutamide has been reported to stimulate LH production which, in turn, increases the testosterone levels in plasma (O'Connor *et al.,* 2002). We believe testosterone could have oxidised into 11-KT instead of undergoing aromatisation to yield E2. However, we did not did not measure the aromatase activity in the present study. Testosterone can be transformed into 11-KT, the biologically active form of androgen in fish even in the absence of gonadotropins (Ueda *et al.,* 1984). It is likely that some signal transduction pathways are initiated by the binding of flutamide to the ARs that lower the circulating levels of E2 (Shilling and Williams 2000). Our hypothesis is supported by a study in which administration of flutamide in diet (50 mg/kg) for 2 wk reduced the E2 levels and increased the 11-KT levels in Eurasian perch (Mandiki *et al.,* 2005). Androgens like testosterone and 11-KT can inhibit the E2 production stimulated by gonadotropins in fish by a cell-surface mediated, non-genomic mechanism

(Braun and Thomas 2003). This pathway would inhibit E2 production to combat increased feminisation (due to E2 exposure) and decreased androgenicity (due to flutamide exposure). Rats administered a dose of 15 mg flutamide per day for 7 days had 42% reduction in hypothalamus-preoptic area aromatase activity (Roselli and Resko 1984).

An induction in the VTG levels as a result of estrogenic exposure in early life stages has been demonstrated in zebrafish (Brion *et al.,* 2004) and fathead minnow (Tyler *et al.,* 1999). Fathead minnows exposed to flutamide 320 µg/L for 7 days or 500 - 1000 µg/L for 21 days and 700 µg/L vinclozolin for 21 days exhibited an increase in the VTG levels (Filby *et al.,* 2007; Jensen *et al.,* 2004; Martinovic *et al.,* 2008; Panter *et al.,* 2004). However, the researchers in these studies also reported either increase in E2 levels (Jensen *et al.,* 2004) or decreased incorporation of VTG into the oocytes (Martinovic *et al.,* 2008). However, we report a higher proportion of early vitellogenic oocytes after exposures to flutamide and/or E2. It should be noted that the E2 concentrations reported in the present study were measured in the head region and do not represent the circulating levels. It is possible that upregulation of ER expression by E2 directly and by flutamide indirectly could have initiated VTG production. Upregulation of the genes for ERs after flutamide exposure has been demonstrated in fish, rats, rabbits, monkeys and humans (Bodker *et al.,* 1997; Filby *et al.,* 2007; Samy *et al.,* 2000; West *et al.,* 1990; Yu *et al.,* 2005). Recently, we found flutamide upregulated the hepatic transcript for ERα and induced VTG protein in the plasma of male Murray rainbowfish (Bhatia *et al.,* 2014). Although, the mechanism with which anti-androgens induce the expression of ER genes remains unclear, a recent study reported down-regulation of ERs after exposure to testosterone in mosquitofish (Huang *et al.,* 2012). It is possible that the increase in the expression of ER transcript could have resulted in the binding

of E2 to the ERs. This could have caused an increase in the VTG and decrease in the E2 levels. However, we did not measure the gene expression of the ERs.

Exposures to flutamide had profound effect on spermatogenesis. Spermatogonial development was impaired and this effect was severe in the presence of E2. In contrast, there was a precocious development of the oocytes with higher proportion of early vitellogenic oocytes after treatment with flutamide and/or E2. There was a small incidence of inter-sex gonads (with more of the ovarian cells) after treatments with E2, Flu high, E2+Flu low and E2+Flu high. Future studies investigating the expression of the genes involved in testicular differentiation (*Dmrt1*, *Dmy*) (Johnsen and Andersen 2012) or ovarian differentiation (*Foxl2*, *aromatase*) (Siegfried and Nuesslein-Volhard 2008) in sexually undifferentiated fish could help better understand the incidences of intersex gonads and sex reversal in sexually undifferentiated fish. Induction in VTG and impairment of the hormonal profile suggested potential reproductive malfunctions. A positive co-relation has demonstrated between the anti-androgenic and estrogenic activities of chemicals using *in vitro* yeast screens, suggesting, anti-androgens can assert estrogenic effects (Grover *et al.,* 2011). In addition, different mechanisms in the body are linked and any deviation from normal in one, can be reflected in the other (Kloas *et al.,* 2009).

CONCLUSIONS

The model anti-androgen, flutamide was designed to treat diseases like prostate cancer in men and polycystic ovarian syndrome in women. The present study confirms previous *in vivo* findings that chemicals identified as anti-androgens like flutamide can interfere with the reproductive fitness in fish. We found that flutamide asserts similar effects as E2 on the biochemical, histological and hormonal profile of juvenile fish. In addition, flutamide did not

significantly increase the E2-induced effects on body-growth; and the concentrations of VTG and sex-steroid hormones. However, qualitative investigation showed that flutamide amplified the E2-induced effects on gonadal cell development in juvenile fish. E2+flu high inhibited the sperm development in the testes and resulted in precocious oocyte development in the ovaries. Based on the previous studies and the data from the present study, we conclude that anti-androgens do not add to the effects of estrogens due to different modes of action. However, they induce similar effects which can cause additive inhibition/stimulation of the gonad development. The incidence of intersex highlights the potential adverse effects of environmentally relevant concentrations of anti-androgens in combination with estrogens on sexual differentiation of juvenile fish. Further research is warranted to elucidate the MoA of flutamide in combination with estrogens at genetic level by investigating the genes involved in gonadal differentiation (Dmrt,1 *Dmy*, *Foxl2* and *aromatase*).

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REFERENCES

Ankley GT, Defoe DL, Kahl MD, Jensen KM, Miracle A, Hartig P, Gray LE, Cardon M, Wilson V. 2004. Evaluation of the model anti-androgen flutamide for assessing the mechanistic basis of responses to an androgen in the fathead minnow (*Pimephales promelas*). *Environ. Sci. Technol.* **38**:6322-6327. 10.1021/es040022b:

Baatrup E, Junge M. 2001. Antiandrogenic pesticides disrupt sexual characteristics in the adult male guppy (*Poecilia reticulata*). *Environ. Health Persp.* **109**:1063-1070. 10.2307/3454962:

Baker DM, Davies B, Dickhoff WW, Swanson P. 2000. Insulin-like growth factor I increases follicle-stimulating hormone (FSH) content and gonadotropin-releasing hormone-stimulated FSH release from coho salmon pituitary cells *in vitro*. *Biol. Reprod.* **63**:865-871. 10.1095/biolreprod63.3.865:

Barske LA, Capel B. 2010. Estrogen represses SOX9 during sex determination in the red-eared slider turtle *Trachemys scripta*. *Dev. Biol.* **341**:305-314. 10.1016/j.ydbio.2010.02.010:

Bayley M, Junge M, Baatrup E. 2002. Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. *Aquat. Toxicol.* **56**:227-239. 10.1016/s0166-445x(01)00210-7:

Bhatia, Kumar A, Du J, Chapman J, McLaughlin MJ. 2013. Di-n-butyl phthalate causes antiestrogenic effects in female murray rainbowfish (*Melanotaenia fluviatilis*). *Environ. Toxicol. Chem.* **32**:2335-44. 10.1002/etc.2304:

Bhatia, Kumar A, Ogino Y, Du J, Gregg A, Chapman J, McLaughlin MJ, Iguchi T. 2014. Effects of the commercial anti-androgen, flutamide on the biomarkers of reproduction in male murray rainbowfish (*Melanotaenia fluviatilis*). *Environ. Toxicol. Chem*. 10.1002/etc.2524:

Bhatia, Kumar A, Ogino Y, Gregg A, Chapman J, McLaughlin MJ, Iguchi T. 2014. Di-n-butyl phthalate causes estrogenic effects in adult male Murray rainbowfish (*Melanotaenia fluviatilis*). *Aquat. Toxicol.* **149C**:103-115. 10.1016/j.aquatox.2014.01.025:

Billard R, Breton B, Richard M. 1981. On the inhibitory effect of some steroids on spermatogenesis in adult rainbow-trout (*Salmo gairdneri*). *Can. J. Zool.* **59**:1479-1487.

Bodker A, Balslev E, Iversen HG, Meyhoff HH, Andersson KE. 1997. The expression of receptors for estrogen and epithelial growth factor in the male rabbit prostate and prostatic urethra following castration. *Scand. J. Urol. Nephrol.* **31**:15-18. 10.3109/00365599709070295:

Bouma J, Cloud JG, Nagler JJ. 2003. In vitro effects of estradiol-17 beta on myoid and fibroblastic cell proliferation in the immature rainbow trout testis. *Fish Physiol. Biochem.* **28**:191-192. 10.1023/B:FISH.0000030524.89979.a5:

Brand T, Slob AK. 1991. Perinatal flutamide and mounting and lordosis behavior in adult female wistar and sprague-dawley rats. *Beh. Brain Res.* **44**:43-51. 10.1016/s0166-4328(05)80238-4:

Braun AM, Thomas P. 2003. Androgens inhibit estradiol-17beta synthesis in Atlantic croaker (*Micropogonias undulatus*) ovaries by a nongenomic mechanism initiated at the cell surface. *Biol Reprod*. **69**:1642-50. 10.1095/biolreprod.103.015479:

Brion F, Tyler CR, Palazzi X, Laillet B, Porcher JM, Garric J, Flammarion P. 2004. Impacts of 17β-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*). *Aquat. Toxicol.* **68**:193-217.

Chakrabarty S, Rajakumar A, Raghuveer K, Sridevi P, Mohanachary A, Prathibha Y, Bashyam L, Dutta-Gupta A, Senthilkumaran B. 2012. Endosulfan and flutamide, alone and in combination, target ovarian growth in juvenile catfish, *Clarias batrachus*. *Comp. Biochem. Physiol.* **155**:491-497. 10.1016/j.cbpc.2011.12.007:

Chen ZL, Lin JM, Uchiyama K, Hobo T. 2000. Determination of critical micelle concentrations of anionic surfactants based on ligand exchange micellar electrokinetic chromatography. *Analytica Chimica Acta*. **403**:173-178.

Collins LL, Lee HJ, Chen YT, Chang M, Hsu HY, Yeh S, Chang C. 2003. The androgen receptor in spermatogenesis. *Cytogen. Genome Res.* **103**:299-301. 10.1159/000076816:

Crowley L, Ivantsoff W, Allen GR. 1986. Taxonomic position of 2 crimson-spotted rainbowfish, *Melanotaenia duboulayi* and *Melanotaenia fluviatilis* (Pisces, Melanotaeniidae), from Eastern Australia, with special reference to their early life-history stages. *Aus. J. Mar. Freshwater Res.* **37**:385-398.

Cyr DG, Eales JG. 1990. Influence of short-term estradiol treatment on plasma thyroid-hormone kinetics in rainbow-trout, *Salmo gairdneri*. *J. Fish Biol.* **36**:391-400. 10.1111/j.1095-8649.1990.tb05619.x:

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. 1998. Identification of estrogenic chemicals in STW effluent - 1 - chemical fractionation and *in-vitro* biological screening. *Environ. Sci. Technol.* **33**:1549-1558.

Filby AL, Thorpe KL, Maack G, Tyler CR. 2007. Gene expression profiles revealing the mechanisms of anti-androgen-and estrogen-induced feminization in fish. *Aquat. Toxicol.* **81**:219-231. 10.1016/j.aquatox.2006.12.003:

Flammarion P, Brion F, Babut M, Garric J, Migeon B, Noury P, Thybaud E, Palazzi X, Tyler CR. 2000. Induction of fish vitellogenin and alterations in testicular structure: preliminary results of estrogenic effects in chub (*Leuciscus cephalus*). *Ecotoxicol.* **9**:127-135.

Gimeno S, Komen H, Gerritsen AGM, Bowmer T. 1998. Feminisation of young males of the common carp, *Cyprinus carpio*, exposed to 4-*tert*-pentylphenol during sexual differentiation. *Environ. Toxicol. Chem.* **43**:77-92.

Goto K, Koizumi K, Takaori H, Fujii Y, Furuyama Y, Saika O, Suzuki H, Saito K, Suzuki K. 2004. Effects of flutamide on sex maturation and behavior of offspring born to female rats treated during late pregnancy. *J. Toxicol. Sci.* **29**:517-34. 10.2131/jts.29.517:

Gray MA, Metcalfe CD. 1997. Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to *p*-nonylphenol. *Environ. Toxicol. Chem.* **16**:1082-1086.

Gray MA, Niimi AJ, Metcalfe CD. 1999. Factors affecting the development of testis-ova in medaka, *Oryzias latipes*, exposed to octylphenol. *Environ. Toxicol. Chem.* **18**:1835-1842. 10.1897/1551-5028(1999)018<1835:fatdot>2.3.co;2:

Grover DP, Balaam J, Pacitto S, Readman JW, White S, Zhou JL. 2011. Endocrine disrupting activities in sewage effluent and river water determined by chemical analysis and in vitro assay in the context of granular activated carbon upgrade. *Chemosphere*. **84**:1512-1520. 10.1016/j.chemosphere.2011.04.032:

Hotchkiss AK, Ankley GT, Wilson VS, Hartig PC, Durhan EJ, Jensen KM, Martinovi D, Gray LE, Jr. 2008. Of mice and men (and mosquitofish): antiandrogens and androgens in the environment. *Biosci.* **58**:1037-1050. 10.1641/b581107:

Huang G-Y, Ying G-G, Liu S, Fang Y-X. 2012. Regulation of reproduction- and biomarker-related gene expression by sex steroids in the livers and ovaries of adult female western mosquitofish (*Gambusia affinis*). *Comp. Biochem. Physiol.* **162**:36-43. 10.1016/j.cbpa.2012.02.006:

Jensen KM, Kahl MD, Makynen EA, Korte JJ, Leino RL, Butterworth BC, Ankley GT. 2004. Characterization of responses to the antiandrogen flutamide in a short-term

reproduction assay with the fathead minnow. *Aquat. Toxicol.* **70**:99-110. 10.1016/j.aquatox.2004.06.012:

Jobling, Burn RW, Thorpe K, Williams R, Tyler C. 2009. Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in English rivers. *Environ. Health Persp.* **117**:797-802. 10.1289/ehp.0800197:

Jobling, Nolan M, Tyler CR, Brighty G, Sumpter JP. 1998. Widespread Sexual Disruption in Wild Fish. *Environ. Sci. Technol.* **32**:2498-2506.

Johnsen H, Andersen O. 2012. Sex dimorphic expression of five dmrt genes identified in the Atlantic cod genome. The fish-specific *dmrt2b* diverged from *dmrt2a* before the fish whole-genome duplication. *Gene*. **505**:221-32. 10.1016/j.gene.2012.06.021:

Kagawa H, Gen K, Okuzawa K, Tanaka H. 2003. Effects of luteinizing hormone and follicle-stimulating hormone and insulin-like growth factor-I on aromatase activity and P450 aromatase gene expression in the ovarian follicles of red seabream, *Pagrus major*. *Biol. Reprod.* **68**:1562-1568. 10.1095/biolreprod.102.008219:

Karels A, Markkula E, Oikari A. 2001. Reproductive, biochemical, physiological, and population responses in perch (*Perca fluviatilis* L.) and roach (*Rutilus rutilus* L.) downstream of two elemental chlorine-free pulp and paper mills. *Environ. Toxicol. Chem.* **20**:1517-1527.

Kassim NM, McDonald SW, Reid O, Bennett NK, Gilmore DP, Payne AP. 1997. The effects of pre- and postnatal exposure to the nonsteroidal antiandrogen flutamide on testis descent and morphology in the Albino Swiss rat. *J. Anatom*. **190**:577-588. 10.1046/j.1469-7580.1997.19040577.x:

Kinnberg K, Toft G. 2003. Effects of estrogenic and antiandrogenic compounds on the testis structure of the adult guppy (*Poecilia reticulata*). *Ecotoxicol. Environ. Safety*. **54**:16-24. Pii s0147-6513(02)00010-6 10.1016/s0147-6513(02)00010-6:

Kiparissis Y, Metcalfe TL, Balch GC, Metcalf CD. 2003. Effects of the antiandrogens, vinclozolin and cyproterone acetate on gonadal development in the Japanese medaka (*Oryzias latipes*). *Aquat. Toxicol.*. **63**:391-403. Pii s0166-445x(02)00189-3 10.1016/s0166-445x(02)00189-3:

Kortenkamp A, Altenburger R. 1998. Synergisms with mixtures of xenoestrogens - a reevaluation using the method of isoboles. *Sci. Total Environ.* **221**:59-73.

KruithofDekker IG, Tetu B, Janssen PJA, VanderKwast TH. 1996. Elevated estrogen receptor expression in human prostatic stromal cells by androgen ablation therapy. *J. Urol.* **156**:1194-1197. 10.1016/s0022-5347(01)65749-5:

Leon A, Wu PS, Hall LC, Johnson ML, Teh SJ. 2008. Global gene expression profiling of androgen disruption in Qurt strain medaka. *Environ. Sci. Technol.* **42**:962-969. 10.1021/es071785c:

Ma D, Chen L, Lui R. 2013. Decrease of antiandrogenic activity in gray water and domestic wastewater treated by the MBR process. *Environ. Sci.* **15**:668-676. 10.1039/c3em30737a:

Makynen EA, Kahl MD, Jensen KM, Tietge JE, Wells KL, Van der Kraak G, Ankley GT. 2000. Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). *Aquat. Toxicol.* **48**:461-475. 10.1016/s0166-445x(99)00059-4:

Mandiki SNM, Babiak I, Bopopi JM, Leprieur F, Kestemont P. 2005. Effects of sex steroids and their inhibitors on endocrine parameters and gender growth differences in

Eurasian perch (*Perca fluviatilis*) juveniles. *Steroids*. **70**:85-94. 10.1016/j.steroids.2004.10.009:

Marchand O, Govoroun M, D'Cotta H, McMeel O, Lareyre JJ, Bernot A, Laudet V, Guiguen Y. 2000. DMRT1 expression during gonadal differentiation and spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*. *Biochimica Et Biophysica Acta-Gene Structure and Expression*. **1493**:180-187. 10.1016/s0167-4781(00)00186-x:

Martinovic D, Blake LS, Durhan EJ, Greene KJ, Kahl MD, Jensen KM, Makynen EA, Villeneuve DL, Ankley GT. 2008. Reproductive toxicity of vinclozolin in the fathead minnow: Confirming an anti-androgenic mode of action. *Environ. Toxicol. Chem.* **27**:478-488. 10.1897/07-206r.1:

Mikkila TFM, Toppari J, Paranko J. 2006. Effects of neonatal exposure to 4-tert-octylphenol, diethylstilbestrol, and flutamide on steroidogenesis in infantile rat testis. *Toxicol. Sci.* **91**:456-466. 10.1093/toxsci/kfj156:

Mills SC, Mourier J, Galzin R. 2010. Plasma cortisol and 11-ketotestosterone enzyme immunoassay (EIA) kit validation for three fish species: the orange clownfish *Amphiprion percula*, the orangefin anemonefish *Amphiprion chrysopterus* and the blacktip reef shark *Carcharhinus melanopterus*. *J. Fish Biol.* **77**:769-777. 10.1111/j.1095-8649.2010.02693.x:

Mylchreest E, Sar M, Cattley RC, Foster PMD. 1999. Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol. Appl. Pharmacol.* **156**:81-95. 10.1006/taap.1999.8643:

Nash JP, Kime DE, Van der Ven LT, Wester PW, Brion F, Maack G, Stahlschmidt-Allner P, Tyler CR. 2004. Long-term exposure to environmental concentrations of the

pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environ. Health Perspect*. **112**:1725-1733.

Nimrod AC, Benson WH. 1998. Reproduction and development of Japanese medaka following an early life stage exposure to xenoestrogens. *Aquatic Toxicology*. **44**:141-156.

O'Connor JC, Frame SR, Ladics GS. 2002. Evaluation of a 15-day screening assay using intact male rats for identifying antiandrogens. *Toxicol. Sci.* **69**:92-108. 10.1093/toxsci/69.1.92:

Ohsako S, Kubota K, Kurosawa S, Takeda K, Qing W, Ishimura R, Tohyama C. 2003. Alterations of gene expression in adult male rat testis and pituitary shortly after subacute administration of the antiandrogen flutamide. *J. Reprod. Dev.* **49**:275-290.

Olivereau M, Leloup J, Deluze A, Olivereau J. 1981. Effect of estradiol on the thyroid-pituitary system of the eel. *Gen. Comp. Endocrinol.* **43**:352-363. 10.1016/0016-6480(81)90295-1:

Panter GH, Hutchinson TH, Hurd KS, Sherren A, Stanley RD, Tyler CR. 2004. Successful detection of (anti-) androgenic and aromatase inhibitors in pre-spawning adult fathead minnows (*Pimephales promelas*) using easily measured endpoints of sexual development. *Aquat. Toxicol.* **70**:11-21. 10.1016/j.aquatox.2004.06.007:

Pask AJ, Calatayud NE, Shaw G, Wood WM, Renfree MB. 2010. Oestrogen blocks the nuclear entry of SOX9 in the developing gonad of a marsupial mammal. *Bmc Biology*. **8** 113

10.1186/1741-7007-8-113:

Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP. 1994. Estrogenic effects of effluents from sewage treatment works. *Chem. Ecol.* **8**:275-285.

Pusey BJ, Arthington AH, Bird JR, Close PG. 2001. Reproduction in three species of rainbowfish (Melanotaeniidae) from rainforest streams in northern Queensland, Australia. *Ecol. Freshwater Fish*. **10**:75-87. 10.1034/j.1600-0633.2001.100202.x:

Rajakumar A, Singh R, Chakrabarty S, Murugananthkumar R, Laldinsangi C, Prathibha Y, Sudhakumari CC, Dutta-Gupta A, Senthilkumaran B. 2012. Endosulfan and flutamide impair testicular development in the juvenile Asian catfish, *Clarias batrachus*. *Aquat. Toxicol.* **110**:123-132. 10.1016/j.aquatox.2011.12.018:

Rasmussen TH, Korsgaard B. 2004. Estrogenic octylphenol affects seminal fluid production and its biochemical composition of eelpout (*Zoarces viviparus*). *Comp. Biochem. Physiol.* **139**:1-10.

Reid HP, Holdway DA. 1995. Early development of the Australian crimson-spotted rainbowfish *Melanotaenia fluviatilis* (Pisces: Melanotaeniidae). *Marine Freshwater Res.* **46**:475-480.

Rolland AD, Lardenois A, Goupil A-S, Lareyre J-J, Houlgatte R, Chalmel F, Le Gac F. 2013. Profiling of androgen response in rainbow trout pubertal testis: relevance to male gonad development and spermatogenesis. *Plos One*. **8** e53302

10.1371/journal.pone.0053302:

Roselli CE, Resko JA. 1984. Androgens regulate brain aromatase-activity in adult male-rats through a receptor mechanism. *Endocrinol.* **114**:2183-2189.

Salierno JD, Kane AS. 2009. 17 alpha-Ethinylestradiol alters reproductive behaviors, circulating hormones, and sexual morphology in male fathead minnows (*pimephales promelas*). *Environ. Toxicol. Chem.* **28**:953-961.

Samy TSA, Schwacha MG, Cioffi WG, Bland KI, Chaudry IH. 2000. Androgen and estrogen receptors in splenic T lymphocytes: Effects of flutamide and trauma-hemorrhage. *Shock*. **14**:465-470.

Schmid AC, Lutz I, Kloas W, Reinecke M. 2003. Thyroid hormone stimulates hepatic IGF-1 mRNA expression in a bony fish, the tilapia *Oreochromis mossambicus*, *in vitro* and *in vivo. Gen. Comp. Endocrinol.* **130**:129-134. Pii s0016-6480(02)00577-4 10.1016/s0016-6480(02)00577-4:

Schulz RW, Bogerd J, Male R, Ball J, Fenske M, Olsen LC, Tyler CR. 2007. Estrogen-induced alterations in amh and dmrt1 expression signal for disruption in male sexual development in the zebrafish. *Environ. Sci. Technol.* **41**:6305-6310. 10.1021/es070785+:

Shanthanagouda AH, Patil JG, Nugegoda D. 2013. Effects of exposure to oestrogenic compounds on aromatase gene expression are gender dependent in the rainbowfish, Melanotaenia fluviatilis. *Comp. Biochem. Physiol.* **157**:162-171. 10.1016/j.cbpc.2012.11.004:

Shilling AD, Williams DE. 2000. The non-aromatizable androgen, dihydrotestosterone, induces antiestrogenic responses in the rainbow trout. *J. Steroid Biochem. Mol. Biol.* **74**:187-194. 10.1016/s0960-0760(00)00122-9:

Siegfried KR, Nuesslein-Volhard C. 2008. Germ line control of female sex determination in zebrafish. *Dev. Biol.* **324**:277-287. 10.1016/j.ydbio.2008.09.025:

Simard J, Luthy I, Guay J, Belanger A, Labrie F. 1986. Characteristics of interaction of the antiandrogen flutamide with the androgen receptor in various target tissues. *Mol. Cell. Endocrinol.* **44**:261-270. 10.1016/0303-7207(86)90132-2:

Sohoni P, Sumpter JP. 1998. Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* **158**:327-339.

Ternes TA, Stumpf M, Mueller J, Haberer K, Wilken RD, Servos M. 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants--I. Investigations in

Germany, Canada and Brazil. *Sci. Total. Environ*. **225**:81-90. S0048-9697(98)00334-9 [pii]:

Tyler CR, van Aerle R, Hutchinsion TH, Maddix S, Trip H. 1999. An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. *Environ. Toxicol. Chem.* **18**:337-347.

Ueda H, Kambegawa A, Nagahama Y. 1984. *In vitro* 11-ketotestosterone and 17-alpha,20-beta-dihydroxy-4-pregnen-3-one production by testicular fragments and isolated sperm of rainbow-trout, *Salmo-gairdneri*. *J. Exp. Zool.* **231**:435-439. 10.1002/jez.1402310318:

van Aerle R, Pounds N, Hutchinson TH, Maddix S, Tyler CR. 2002. Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow, *Pimephales promelas*. *Ecotoxicol.* **11**:423-34.

Van den Belt K, Wester PW, Van der Ven LTM, Verheyen R, Witters H. 2002. Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): Time dependency and reversibility. *Environ. Toxicol. Chem.* **21**:767-775.

Velasco-Santamaria YM, Madsen SS, Bjerregaard P, Korsgaard B. 2010. Effects of 17 beta-trenbolone in male eelpout *Zoarces viviparus* exposed to ethinylestradiol. *Analyt. Bioanalyt. Chem.* **396**:10.1007/s00216-009-3262-2:

Wang R-S, Yeh S, Tzeng C-R, Chang C. 2009. Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. *Endocrine Rev.* **30**:119-132. 10.1210/er.2008-0025:

West NB, Chang C, Liao S, Brenner RM. 1990. Localization and regulation of estrogen, progestin and androgen receptors in the seminal-vesicle of the rhesus-monkey. *J. Steroid Biochem. Mol. Biol.* **37**:11-21. 10.1016/0960-0760(90)90367-t:

Wester PW, Canton JH, Bisschop A. 1985. Histopathological study of *Poecilia reticulata* (guppy) after long-term ß-hexachlorocyclohexane exposure. *Aquat. Toxicol.* **6**:271-296.

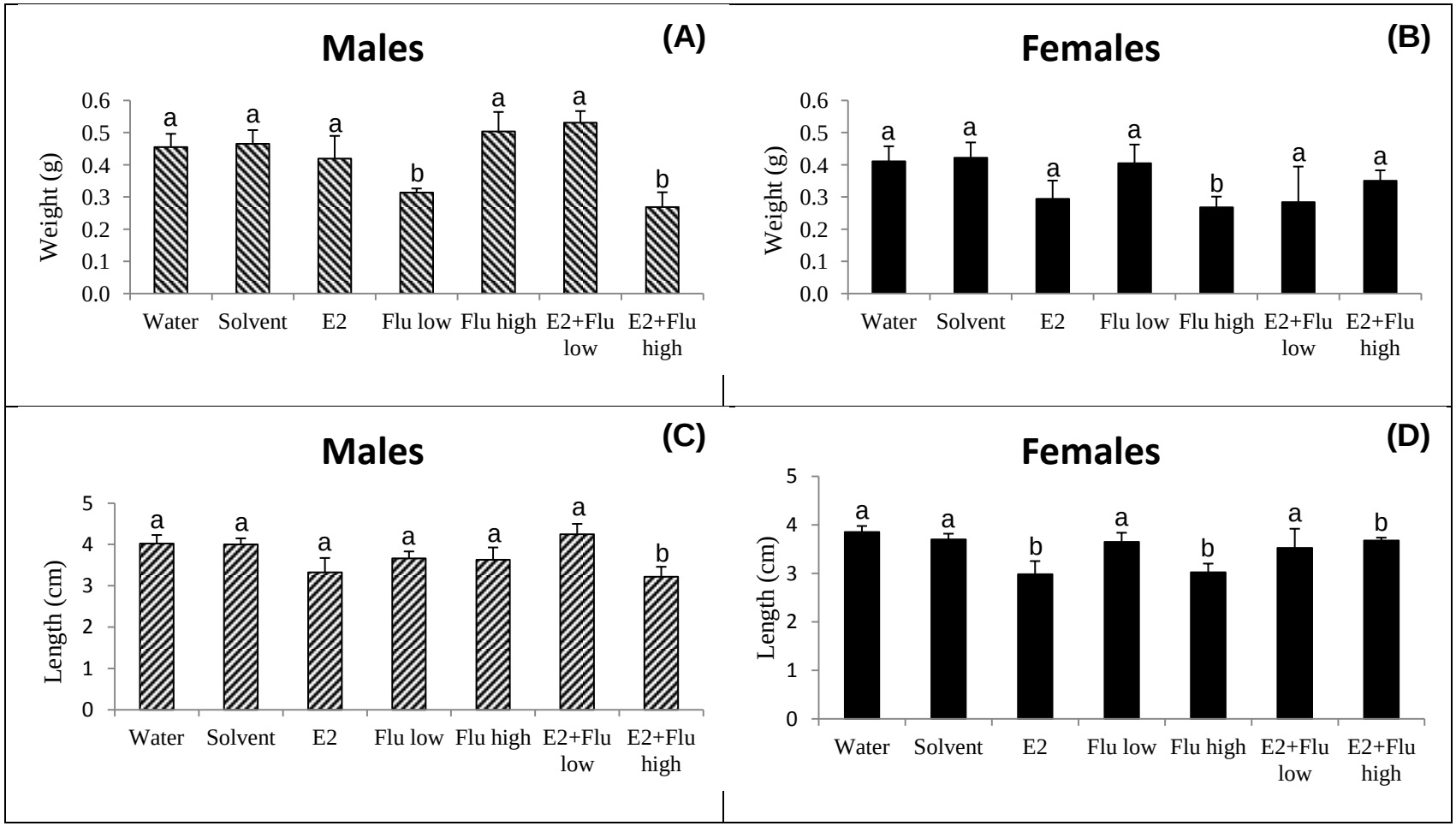
Woods M, Kumar A. 2011. Vitellogenin induction by 17beta-Ethynylestradiol in male Murray rainbowfish (*Melanotaenia fluviatilis*). *Environ. Toxicol. Chem.* **30**:2620-2627. 10.1002/etc.660:

Yeh SY, Tsai MY, Xu QQ, Mu XM, Lardy H, Huang KE, Lin H, Yeh SD, Altuwaijri S, Zhou XC, Xing LP, Boyce BF, Hung MC, Zhang S, Gan L, Chang CS. 2002. Generation and characterization of androgen receptor knockout (ARKO) mice: An in vivo model for the study of androgen functions in selective tissues (vol 99, pg 13498, 2002). *Proceed. Nat. Acad. Sci.* **99**:15245-15245. 10.1073/pnas.242581399:

Yu HP, Yang SL, Choudhry MA, Hsieh YC, Bland KI, Chaudry IH. 2005. Mechanism responsible for the salutary effects of flutamide on cardiac performance after trauma-hemorrhagic shock: Upregulation of cardiomyocyte estrogen receptors. *Surgery*. **138**:85-92. 10.1016/j.surg.2005.03.006:

Zaroogian G, Gardner G, Horowitz DB, Gutjahr-Gobell R, Haebler R, Mills L. 2001. Effect of 17β-estradiol, *o,p′*-DDT, octylphenol and *p,p′*-DDE on gonadal development and liver and kidney pathology in juvenile male summer flounder (*Paralichthys dentatus*). *Aquat. Toxicol.* **54**:101-112.

Zhao J-L, Ying G-G, Yang B, Liu S, Zhou L-J, Chen Z-F, Lai H-J. 2011. Screening of multiple hormonal activities in surface water and sediment from the Pearl River system, South China, using effect-directed in vitro bioassays. *Environ. Toxicol. Chem.* **30**:2208-2215. 10.1002/etc.625: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Weight (g)
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0.4
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0.2
0.1
0.0
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a
b
a
a
b
Water
Solvent
E2
Flu low
Flu high
E2+Flu low
E2+Flu high
Females
(B)
Weight (g)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
a
b
a
a
Water
Solvent
E2
Flu low
Flu high
E2+Flu low
E2+Flu high
Males
(C)
Length (cm)
5
4
3
2
1
0
a
a
a
a
a
a
b
Water
Solvent
E2
Flu low
Flu high
E2+Flu low
E2+Flu high
Females
(D)
Length (cm)
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4
3
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E2+Flu high

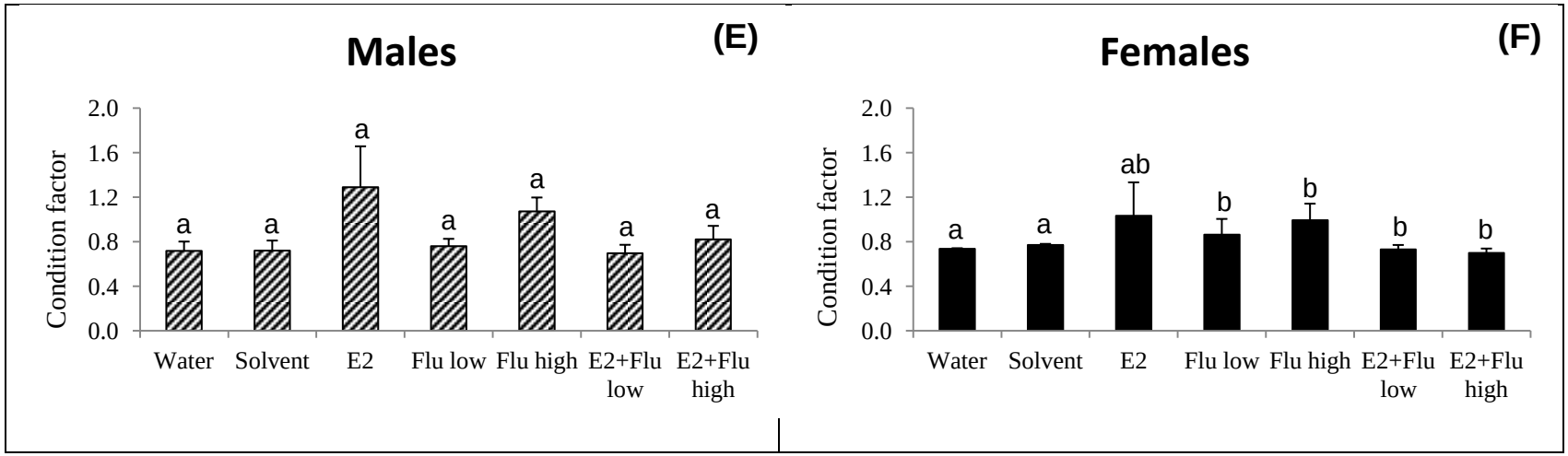


Figure 1: Weight of (A) male and (B) female; length of (C) male and (D) female and condition factor of (E) male and (F) juvenile Murray rainbowfish after exposures to flutamide and/or 17β-estradiol for 35 days. Bars represent standard error.

Means not followed by the same letter are significantly different ($p \leq 0.05$)

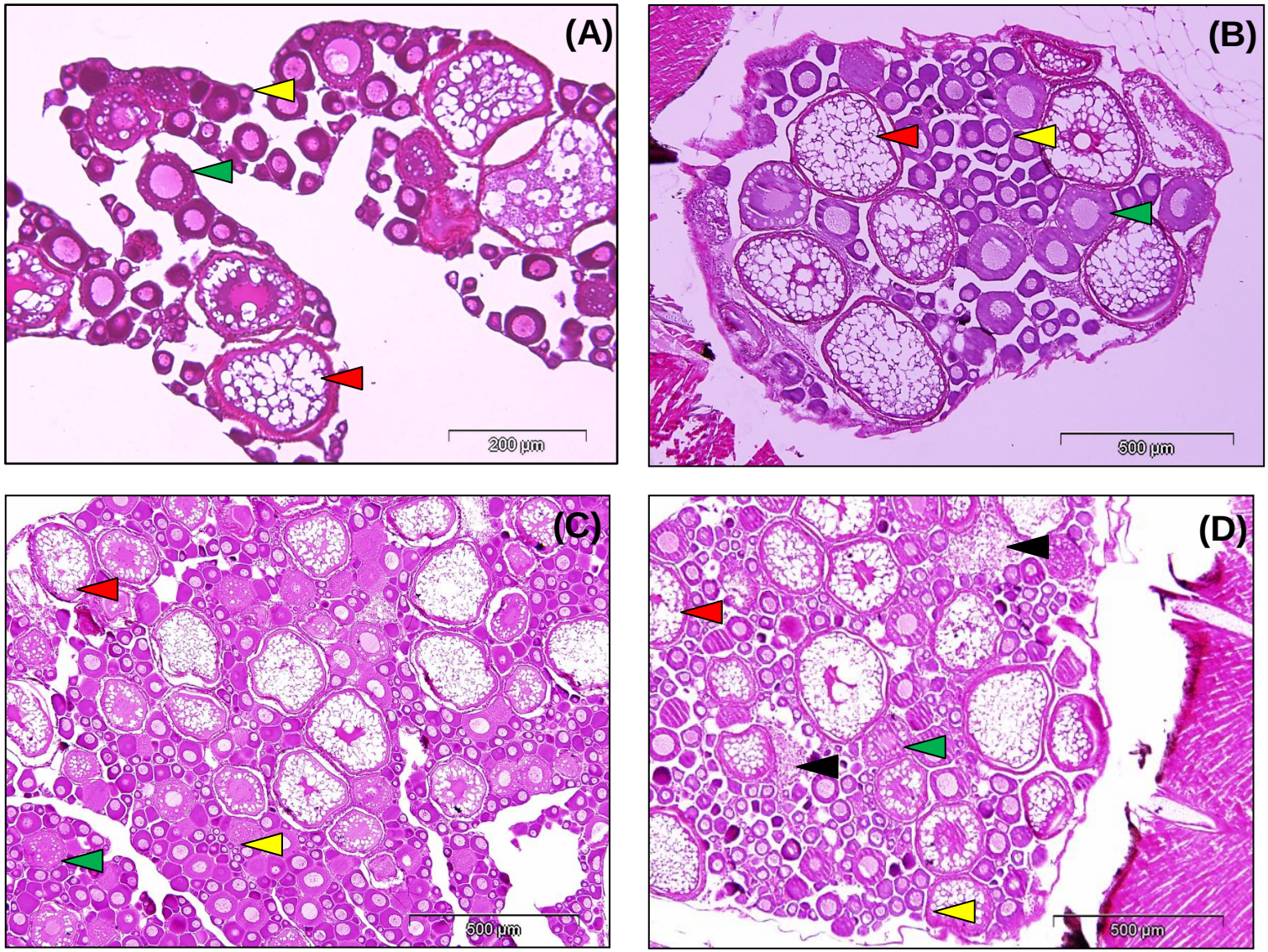

(A)
200 µm
(B)
500 µm
(C)
500 µm
(D)
500 µm

Figure 2: Photomicrographs of the longitudinal sections of the abdominal regions of juvenile Murray rainbowfish (*Melanotaenia fluviatilis*) showing the ovaries. A) Water control B) 25 ng/L E2 (E2) C) 250 µg/L flutamide (Flu high) D) 25 ng/L E2 + 250 µg/L flutamide (E2+Flu high). The arrows represent perinucleolar oocytes (◁), cortical alveolar oocytes (◁), early vitellogenic oocytes (◁) and interstitial fibrosis (◁). Note the increase in proportion of early vitellogenic oocytes in the E2, Flu high and E2+Flu high treatments and the interstitial fibrosis in E2+Flu high treatment.

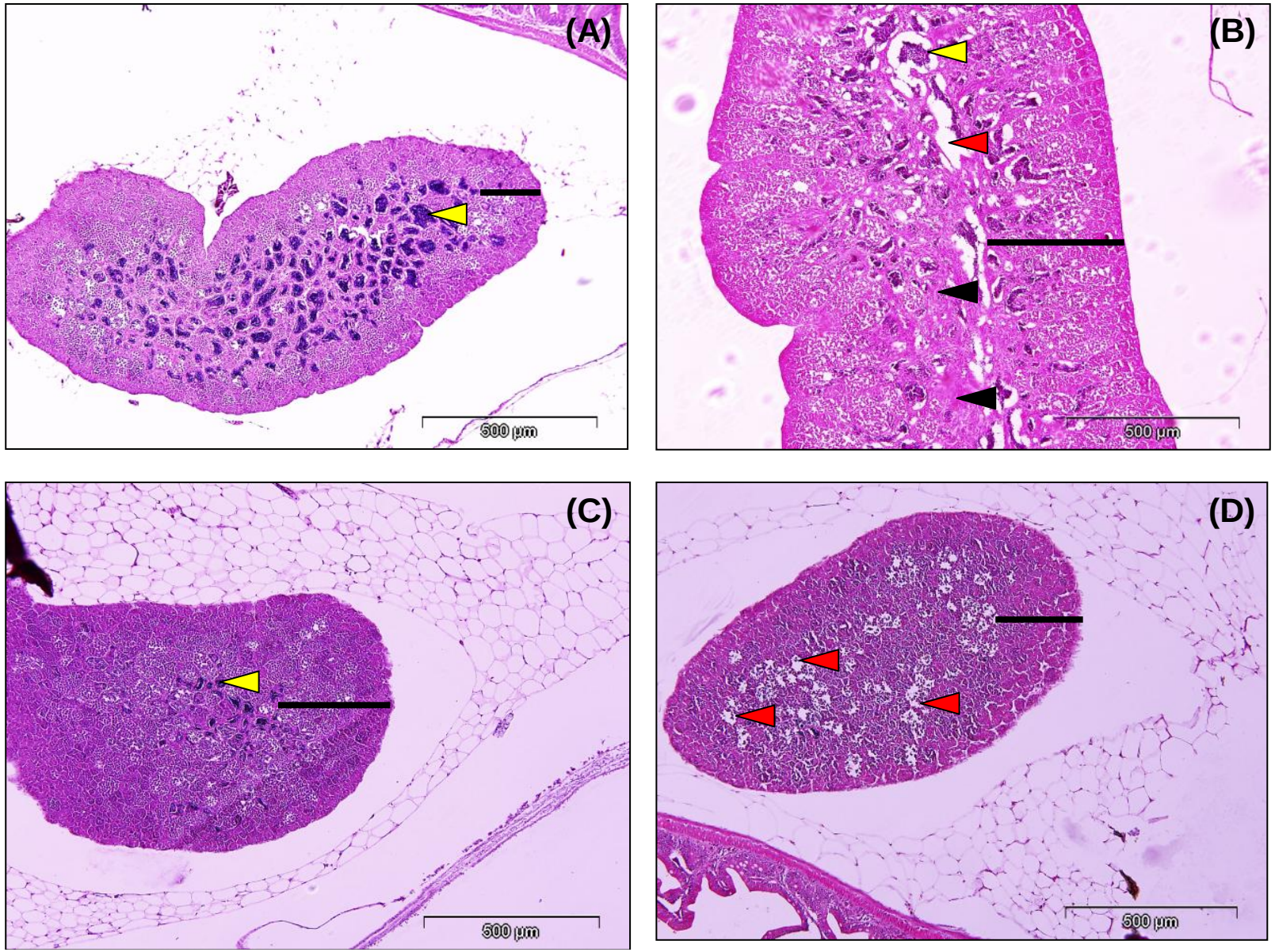

(A)
500 µm
(B)
500 µm
(C)
500 µm
(D)
500 µm

Figure 3: Photomicrographs of the longitudinal sections of the abdominal regions of juvenile Murray rainbowfish (*Melanotaenia fluviatilis*) showing the testes. A) Water control B) 25 ng/L E2 (E2) C) 250 µg/L flutamide (Flu high) D) 25 ng/L E2 + 250 µg/L flutamide (E2+Flu high). The arrows represent spermatozoa (), vacuolation (and interstitial fibrosis ( ). Note the increase in the height of germinal epithelium represented by (in E2, Flu high and E2+Flu high treatments. Note the reduction in the proportion of sperms in E2 and Flu high treatments and absence of sperms in E2+Flu high treatment.

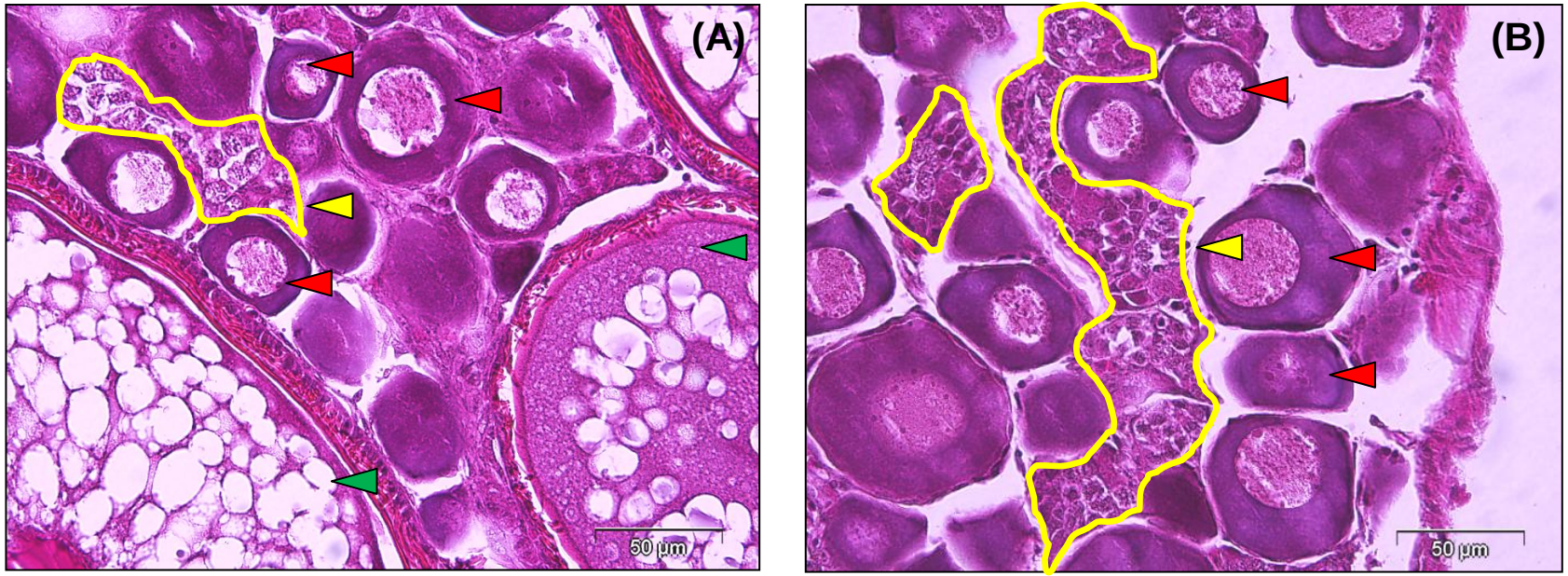
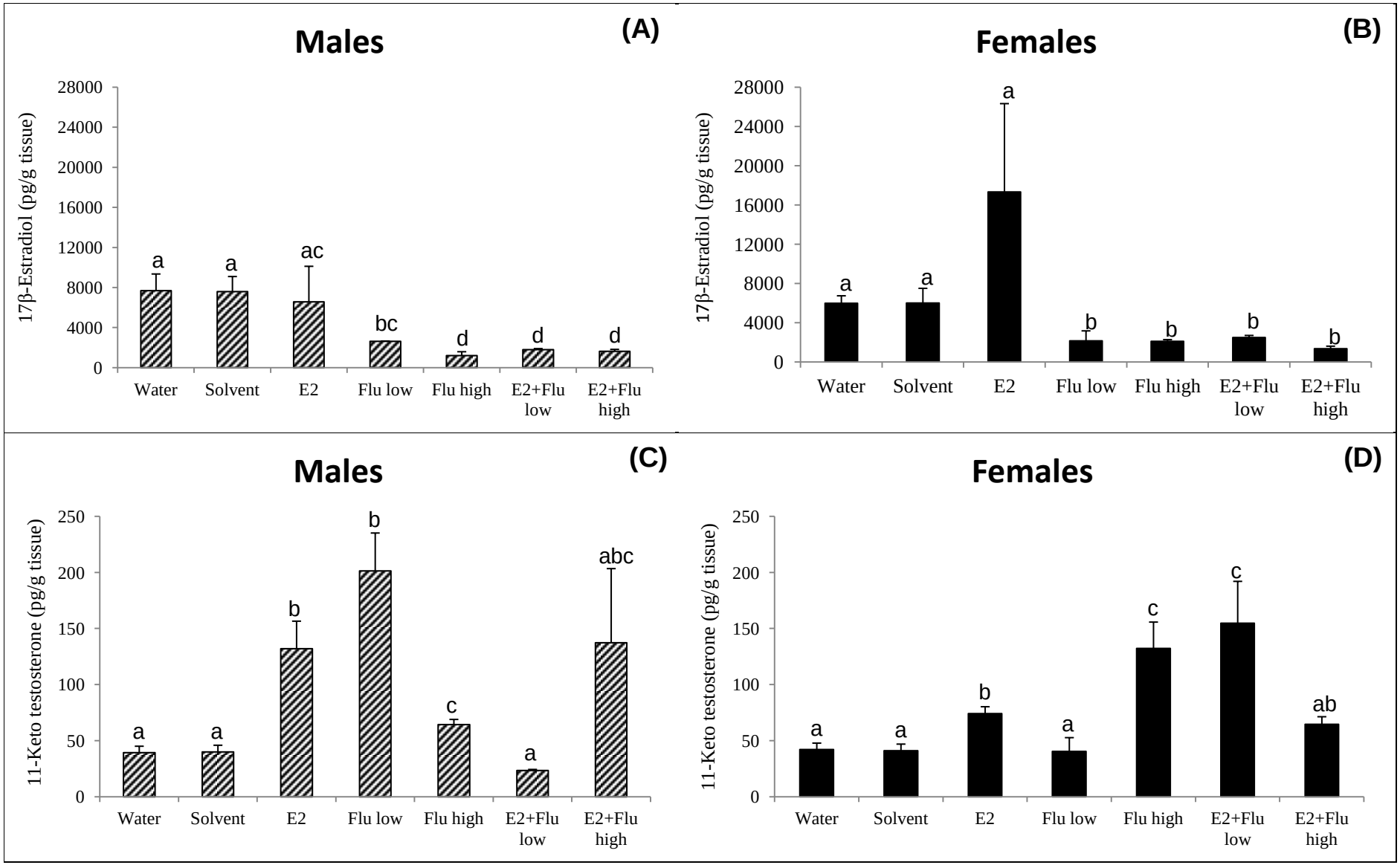


Figure 4: Photomicrographs of the longitudinal sections of the abdominal regions of juvenile Murray rainbowfish (*Melanotaenia fluviatilis*) showing the intersex gonads in fish after treatment with A) 250 µg/L flutamide (Flu high) and B) 25 ng/L E2 + 250 µg/L flutamide (E2+Flu high). The arrows represent perinucleolar oocytes ( ) and early vitellogenic oocytes ( ). Note the development of spermatocytes ( ) between the oocytes.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17β-Estradiol (pg/g tissue)
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24000
20000
16000
12000
8000
4000
0
a
a
ac
bc
d
d
d
Water
Solvent
E2
Flu low
Flu high
E2+Flu low
E2+Flu high
Females
(B)
17β-Estradiol (pg/g tissue)
a
a
a
b
b
b
b
Males
(C)
11-Keto testosterone (pg/g tissue)
250
200
150
100
50
0
a
a
b
b
c
a
abc
Females
(D)
11-Keto testosterone (pg/g tissue)
a
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b
a
c
c
ab

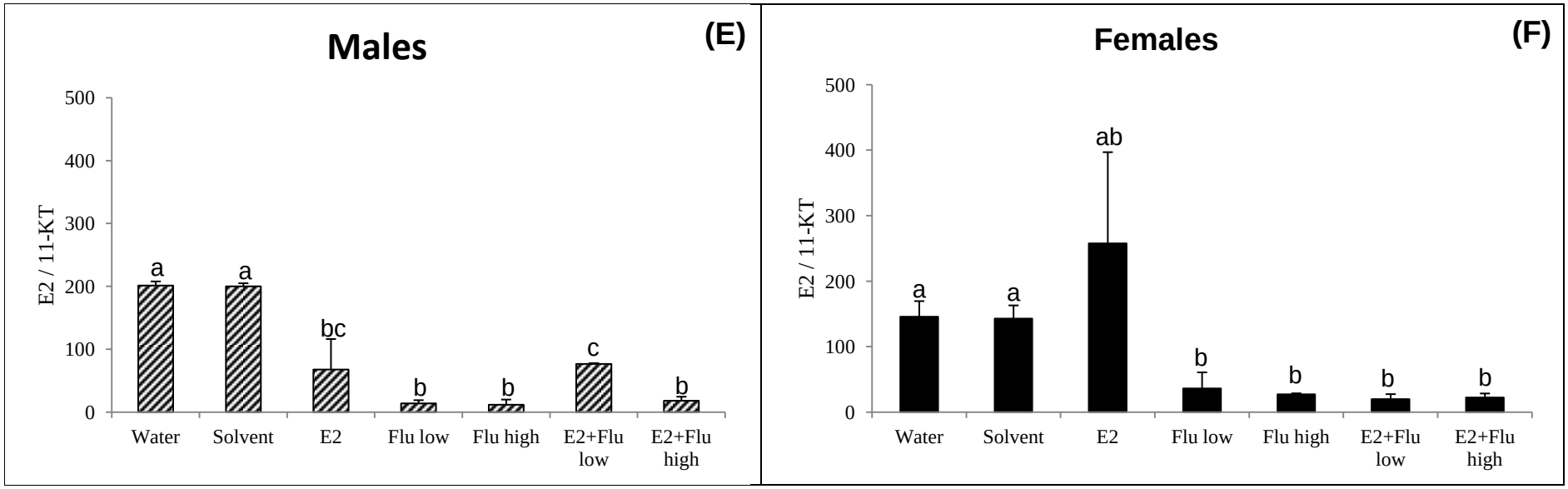


Figure 5: Head concentrations of 17β-estradiol (E2) in (A) males and (B) females; 11-keto testosterone (11-KT) in (C) males and (D) females; E2/11-KT value in (E) males and (F) females of Murray rainbowfish after treatment with 25 ng/L E2 (E2), 25 µg/L flutamide (Flu low), 250 µg/L flutamide (Flu high), 25 ng/L E2 + 25 µg/L flutamide (E2+Flu low) and 25 ng/L E2 + 250 µg/L flutamide (E2+Flu high). Bars represent standard error.

Means not followed by the same letter are significantly different ($p \leq 0.05$).

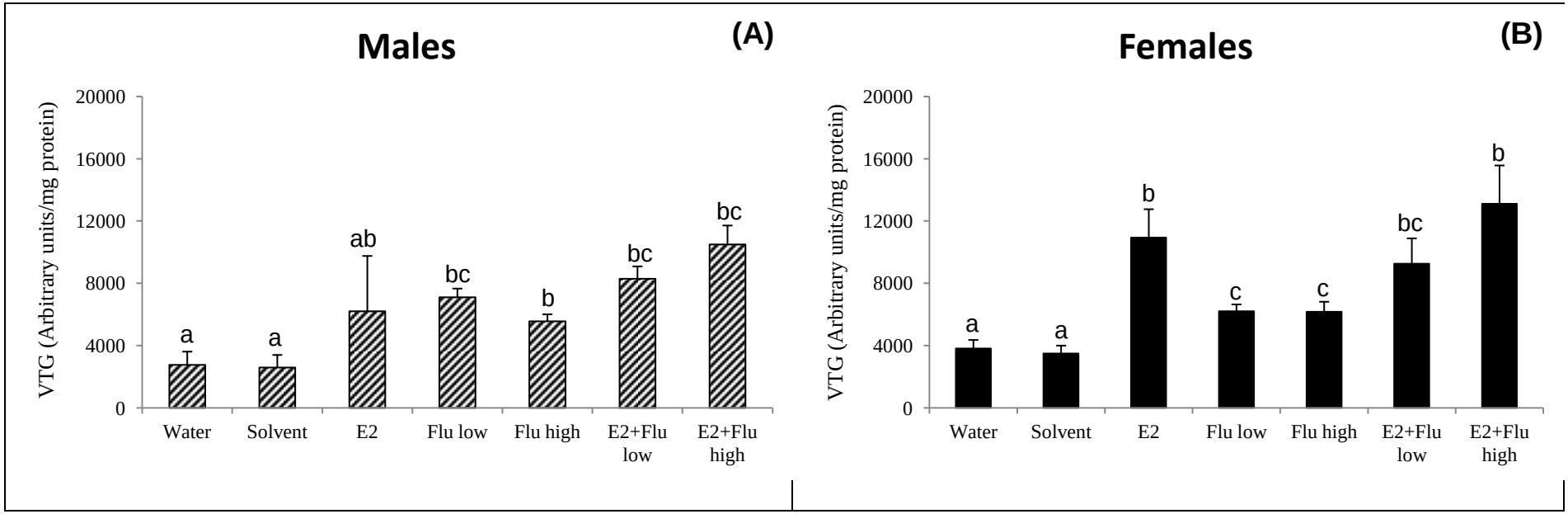


Figure 6: Tail concentrations of vitellogenin (VTG) in (A) males and (B) females of Murray rainbowfish after treatment with 25 ng/L E2 (E2), 25 µg/L flutamide (Flu low), 250 µg/L flutamide (Flu high), 25 ng/L E2 + 25 µg/L flutamide (E2+Flu low) and 25 ng/L E2 + 250 µg/L flutamide (E2+Flu high). Bars represent standard error.

Means not followed by the same letter are significantly different ($p \leq 0.05$)

Supplementary Table 1: Measured concentrations (mean ± SE) of 17β-estradiol and flutamide in fish tanks by GCMS-MS and LCMS-MS, respectively before the start of exposure and 24 h after spiking of the test solutions and water quality parameters (mean ± SE) measured daily in fish tanks (n = 4)

Treatment	Measured concentrations				Water quality			Temperature (°C)
	17β-estradiol (ng/L)		Flutamide (µg/L)		pH	Dissolved oxygen (%)	Conductivity (µS/cm)	
	Before the start of exposure	24 h after spiking	Before the start of exposure	24 h after spiking				
Water	<LOD[d]	<LOD[d]	<LOD[e]	<LOD[e]	6.8 ± 0.6	82 ± 5	1187 ± 95	23 ± 0.1
Solvent	<LOD[d]	<LOD[d]	<LOD[e]	<LOD[e]	6.8 ± 0.6	82 ± 3	1211 ± 98	
E2[a]	22 ± 11	14 ± 1	<LOD[e]	<LOD[e]	7.0 ± 0.6	82 ± 5	1214 ± 98	
Flu low[b]	<LOD[d]	<LOD[d]	23 ± 3	19 ± 1	6.9 ± 0.6	83 ± 6	1185 ± 95	
Flu high[c]	<LOD[d]	<LOD[d]	263 ± 12	212 ± 8	6.8 ± 0.5	84 ± 5	1207 ± 97	
E2[a] + Flu low[b]	29 ± 2	20 ± 1	28 ± 1	23 ± 2	6.9 ± 0.6	83 ± 4	1235 ± 92	
E2[a] + Flu high[c]	26 ± 2	21 ± 2	257 ± 7	223 ± 10	7.1 ± 0.5	82 ± 4	1251 ± 89	

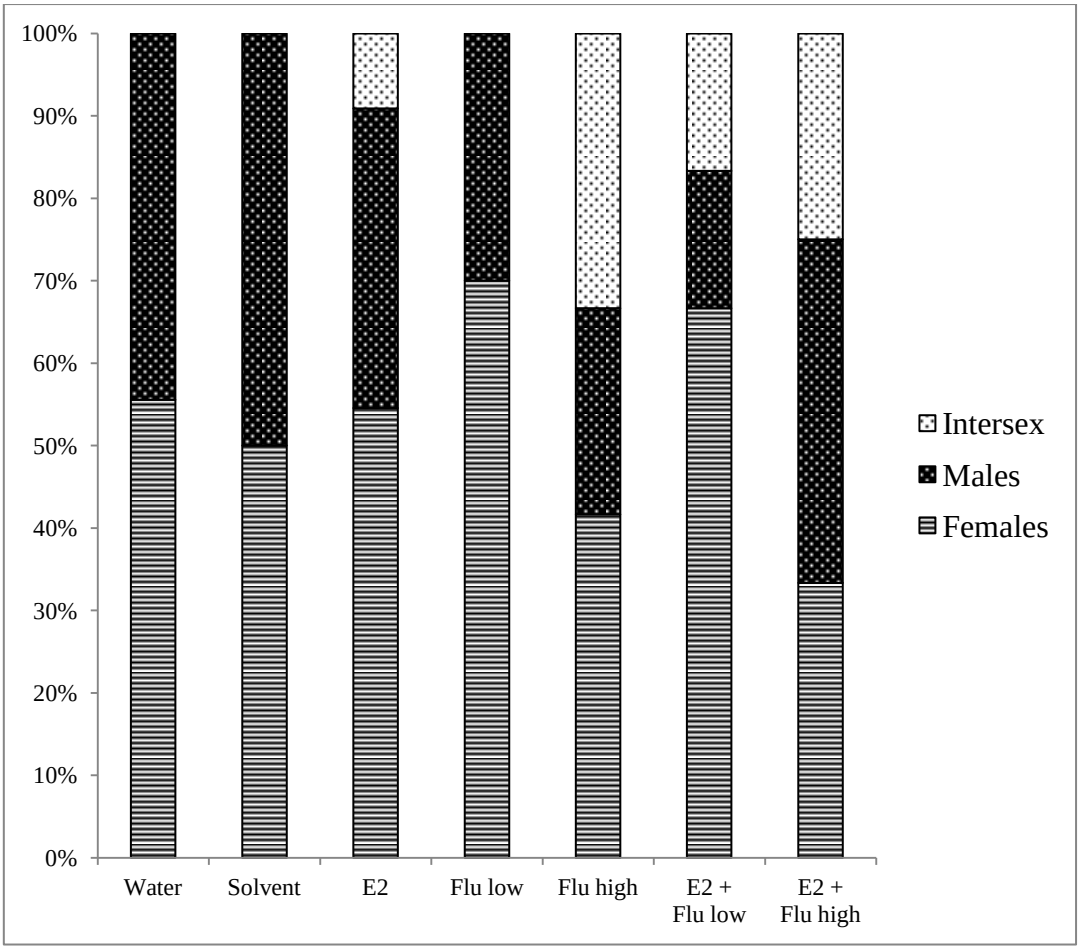
[a]Nominal concentration of 17β-estradiol 25 ng/L

[b]Nominal concentration of flutamide low 25 µg/L

[c]Nominal concentration of flutamide high 250 µg/L

[d]Limit of detection of 17β-estradiol 2 ng/L

[e]Limit of detection of flutamide 10 ng/L



Supplementary Figure 1: Proportions of male, female and intersex juvenile Murray rainbowfish after exposures to 25 ng/L E2 (E2), 25 µg/L flutamide (Flu low), 250 µg/L flutamide (Flu high), 25 ng/L E2 + 25 µg/L flutamide (E2+Flu low) and 25 ng/L E2 + 250 µg/L flutamide (E2+Flu high) for 35 days. There were no significant differences in the ratios of different sexes across the treatments.

CHAPTER 5

Di-n-butyl phthalate causes estrogenic effects in adult male Murray rainbowfish (*Melanotaenia fluviatilis*).

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CHAPTER 6

Di-n-butyl phthalate causes antiestrogenic effects in female Murray rainbowfish (*Melanotaenia fluviatilis*)

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CHAPTER 7

Long-term exposures to di-n-butyl phthalate inhibit body growth and impair gonad development in juvenile Murray rainbowfish (*Melanotaenia fluviatilis*)

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Long-term exposures to di-n-butyl phthalate inhibit body growth and impair gonad development in juvenile Murray rainbowfish (*Melanotaenia fluviatilis*)

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ABSTRACT

The aim of the present study was to evaluate whether long-term exposures to environmentally relevant concentrations of di-n-butyl phthalate (DnBP) disrupt the reproduction-based endpoints in juvenile Murray rainbowfish (*Melanotaenia fluviatilis*). Fish were exposed to 5, 15 or 50 µg/L DnBP for 30, 60 and 90 days each, and the effects on survival; body growth; whole-body concentrations of sex steroid hormones; and gonadal development were investigated. The lowest observed effective concentration to affect the condition factor after 90 days was 5 µg/L. Complete feminisation of the gonad was noted in fish exposed to 5 µg/L for 90 days and to 15 and 50 µg/L of DnBP for 30 or 60 days. After 90 days of exposure to DnBP, the ovaries were regressed and immature as opposed to the control fish which were in early-vitellogenic stage. Testes, present only in fish exposed to 5 µg/L of DnBP for 30 or 60 days, were immature in comparison to the control fish that contained testes in mid-spermatogenic phase. The E2/11-KT ratio was significantly higher only after exposures to 5 µg/L of DnBP for 90 days and 50 µg/L of DnBP for 30 days. Our

data suggest that long-term exposures to 5 µg/L of DnBP for 30 days did not have profound effects on body growth and gonadal differentiation of fish. However, 30 days of exposure to 15 µg/L could interfere with the gonad development and to 50 µg/L could compromise the hormonal profile of juvenile fish.

Keywords: Sexually-undifferentiated fish; phthalates; chronic exposure; gonadal differentiation; steroid hormones

INTRODUCTION

Phthalic acid esters or phthalates were first created in the early twentieth century for application as industrial plasticisers in the manufacture of products like inks, medicines, cosmetics, toys etc. The current global annual production of phthalates is 6 million tonnes (www.phthalates.com). Since phthalates do not bind chemically to the substrates, they can leach out of the consumer products and diffuse into the environment (Autian 1973). Due to their high production volume and continuous release into the environment, phthalates are regularly detected in aquatic ecosystems. The concentration of one of the most commonly used phthalate, di-n-butyl phthalate (DnBP) detected in freshwater around the world is highly variable ranging from ng/L to mg/L – in Austria (2.4 ng/L), Australia (46.4 ng/L), Japan (540 ng/L), China (3.60 µg/L), UK (12 – 33.5 µg/L), Netherlands (250 µg/L), Germany (500 µg/L) and South Africa (10.2 mg/L) (Clara *et al.,* 2010; Fatoki *et al.,* 2010; Fatoki and Vernon 1990; Fromme *et al.,* 2002; Suzuki *et al.,* 2001; Tan *et al.,* 2007; Vethaak *et al.,* 2005; Zeng *et al.,* 2008).

In vitro assays have reported anti-androgenic activity of DnBP, with IC_{50} value of 1.05×10^{-6} M (Shen *et al.,* 2009). With the detection of phthalates in the receiving

environment world-wide, their potential threat to the reproductive health of male fish has been investigated by some environmental toxicologists. For instance, Aoki and co-workers reported anti-androgenic effects, as evidenced by decrease in spiggin production in adult male three-spined stickleback (*Gasterosteus aculeatus*) after exposures to 35 µg/L DnBP for 22 days (Aoki *et al.,* 2011). Exposure of male zebrafish (*Danio rerio*) to 5000 mg DEHP/kg of body weight disrupted spermatogenesis and reduced the proportion of spermatozoa (Uren-Webster *et al.,* 2010). Testicular growth was inhibited in carp (*Cyprinus carpio*) treated with diethyl phthalate (DEP) (Barse *et al.,* 2007). In the estrogen receptor (ER)-mediated reporter gene assay, DnBP was demonstrated to be estrogenic at concentration of 1×10^{-4} M and induced cumulative estrogenicity in the presence of endogenous estrogens (Harris *et al.,* 1997; Jobling *et al.,* 1995; Shen *et al.,* 2009). The estrogenic potency of phthalates was recently confirmed in *in vivo* studies using male fish. Short-term exposures to high concentration of phthalates induced the circulating levels of the egg yolk-precursor protein, vitellogenin (VTG) or the expression of its gene in liver of male fish (Bhatia *et al.,* 2014; Uren-Webster *et al.,* 2010). Interestingly, phthalates have also been demonstrated to induce anti-estrogenicity in female fish. For instance, reduction in 17β-estradiol (E2) concentrations in female carp exposed to 20.5 mg diethylhexyl phthalate (DEHP) for 48 h (Han *et al.,* 2009), decrease in fecundity in female zebrafish after treatment with 40 µg/L DEHP for 3 wk (Carnevali *et al.,* 2010) and decline in VTG protein in female Murray rainbowfish (*Melanotaenia fluviatilis*) exposed to 1000 µg/L DnBP for 7 d (Bhatia *et al.,* 2013).

A growing body of mammalian studies have reported feminizing effects (like retention of nipples and reduction in anogenital distance) and anti-androgenic effects (like malformations of epididymis and vas deferens, cryptorchidism and hypospadias) in male rats administered DnBP during critical stages of their development (Foster *et al.,* 2001; Kim *et*

al., 2004; Mylchreest *et al.,* 2000; Wolf *et al.,* 1999). Since mammals and fish share similar vertebrate etiology, it is hypothesised that the same endocrine disruptors like phthalates can compromise the reproductive fitness in juvenile fish as well. In comparison to mammals, fish have a larger sensitive window of sexual differentiation which continues in juvenile stage of the life-cycle. Therefore, exposures to endocrine disrupting chemicals (EDCs) during juvenile stages can cause endocrine disruption or even sex reversal. For instance, inter-sex gonads were induced in medaka (*Oryzias latipes*) after treatment with anti-androgens like cyperoterone acetate and vinclozolin and estrogens like nonyl phenol and E2 (Gray and Metcalfe 1997; Nimrod and Benson 1998). In a recent study, exposure of European pikeperch (*Sander lucioperca*) to up to 2 g of DnBP per kg feed during the sex differentiation period (days 61 – 96 post hatch) delayed testicular development (Jarmolowicz *et al.,* 2013). Despite their widespread and long-term usage, demonstrated ability to interfere with reproductive development during the sensitive phases of life-cycle in mammals and ubiquitous detection in freshwater, phthalates have not been extensively tested for their effects after long-term exposures in juvenile fish.

In the present study, the endocrine disrupting-potencies of exposures for long-term to environmentally relevant concentrations of DnBP were assessed using gonadal histopathology and the whole-body concentrations of the sex steroid hormones as the responses in juvenile Murray rainbowfish. The Murray rainbowfish (family Melanotaeniidae) is a native Australian fish species residing in Murray-Darling river system. The embryonic development is completed in 7 – 9 days and the larvae (3 – 5 mm) grow into adults in 6 – 8 months. We have recently reported the effects of DnBP on the biomarkers of reproduction in adult male and female fish of this species (Bhatia *et al.,* 2013; Bhatia *et al.,* 2014). Based on the fresh-water recommendations of DnBP in Australia (> 9.9 µg/L)

(http://www.environment.gov.au/resource/australian-and-new-zealand-guidelines-fresh-and-marine-water-quality-volume-1-guidelines), we aimed to investigate the effects of exposures of 5 µg/L (low), 15 µg/L (moderate) and 50 µg/L (high) of DnBP for 30, 60 and 90 days on the reproduction-based endpoints in sexually undifferentiated Murray rainbowfish.

MATERIALS AND METHODS

Culture and maintenance of fish

Sexually undifferentiated Murray rainbowfish (30 days post hatch [dph]) (10 mg approximately) were obtained from Aquarium Industries, Victoria. Fish were acclimated to the laboratory environment for 2 weeks in artificial freshwater (7.5 g $MgSO_4$, 9.6 g $NaHCO_3$, 1.5 g $CaCl_2$, 40 g NaCl, 3 g KCl and 6 g $CaSO_4$ in 100 L Milli-Q water at pH 6.8 – 7.5; conductivity 1200 – 1500 µS/cm; dissolved oxygen [DO] > 60%) at loading <0.5 g/L. During this period, the temperature in the laboratory was maintained at 23°C with 16 h:8 h light:dark photoperiod and 60 min gradual sunrise/sunset. Fish were fed 4% (w/w) baby brine shrimp from frozen stocks daily. The maintenance and testing of the fish were in accordance to animal ethics approvals (S-2010-084 – The University of Adelaide; 774-12/13 – CSIRO Food Sciences and Nutrition, Adelaide).

Chemicals

Di-n-butyl phthalate (DnBP; CAS number 84-74-2) (1,2-dibenzenecarboxylic acid) was purchased from Sigma-Aldrich. Stock solution (100 g/L) was prepared in ultra-pure methanol in amber glass bottle and kept at -20°C in dark until use. Working solutions (1 – 10 g/L) were prepared daily from the stock solution. Fresh stock solution was prepared every week.

Fish exposure

Fish were exposed in 1 L water in glass beakers to 5, 15 and 50 µg/L DnBP in a semi-static system (60 beakers in total). Water control and 0.0005% solvent control beakers were also set up. The concentration of the solvent was same across all DnBP treatments and the solvent control. Four beakers containing four fish in each were used (16 fish per treatment per time interval with 240 total fish). After 30 days of exposure, the fish were transferred to beakers containing 2 L water to account for their growth. Oxygenation with very light bubbling was set up using capillaries attached to the aerators. The temperature was maintained at 23°C and recorded every 5 s using a logger (Hobo) placed in one of the testing beakers. The physical conditions (temperature and light intensity in the laboratory; and DO, pH and conductivity of water) during the test were similar to those during acclimation period. Fish were fed 4% (w/w) baby brine shrimp once daily. The water in the testing beakers was renewed and spiked with fresh DnBP solutions daily. The conductivity, DO and pH of the water were measured every day just before renewals.

Sampling procedure

After 30 days of exposure to DnBP, 16 fish from each treatment were anaesthetised in 20 mg/L tricaine methanesulfonate (MS222). The lengths and weights of the fish were measured and the condition factor (CF) was calculated according to the formula, CF = body weight (g) / [total length (cm)]^3 x 100. Eight fish were snap-frozen and stored separately at -80°C until use for whole-body hormone analyses. The remaining eight fish were fixed in 10% neutral buffered formalin for investigation of gonad development by whole body sectioning. The same procedure was adopted for sampling after 60 and 90 days of the test. Due to small size of the fish (1 – 2 cm), the present study was designed to conduct whole body hormone and histological analyses.

Water chemistry

Two samples of water (500 mL) were collected from the control and from the 5, 15 and 50 µg/L of DnBP treatments before the start of the exposure (0 day samples). Water (500 mL) was also collected on days 1, 7, 14, 21, 35, 42, 49, 56, 63, 70, 77, 84 and 90 of the exposure (24 h after spiking) from all the 4 replicates in each treatment. The glass amber bottles used to collect water were washed in hexane, acetone and Pyroneg before baking at 400°C for 8 h to prevent contamination by phthalates. The water was spiked with 0.01 M of sulphuric acid to prevent degradation of DnBP by microbial growth and filtered through glass filters with pore sizes 1.2 µm and 0.3 µm (Advantec). The filtered water was then loaded onto cartridges (Oasis), pre-conditioned with ultra-pure water and methanol. The solid phase was extracted from the water and eluted two times using methanol and dichloromethane (3 mL each). The solvents were evaporated under a gentle stream of ultra-pure nitrogen gas at 30°C and the solid phase extract was reconstituted in 1 mL hexane.

The concentrations of DnBP in water were measured using gas chromatography-mass spectrometry (GC-MS) (Network mass selective detector, Agilent) according to the method of Bhatia et al. (2013). Sample (1 µL) was injected into the GC-MS column using ultra-pure helium as the carrier gas at 280°C. A flow rate of 49.6 mL/min was maintained. Quantitative determinations were performed in the single-ion monitoring mode. The concentrations of DnBP in the samples were measured based on its retention time and the relative abundance of target ions. The DnBP standards (0.06 µg/L to 62.5 µg/L) were run at the beginning and at the end of the entire sequence of the samples and one standard was analysed after running three samples to ensure the accuracy of the instrument. The limit of quantification (LOQ) was

set as the concentration of DnBP with response area 20% of the lowest concentration of the standard. The limit of detection (LOD) was set as one-third of the LOQ.

Histological examination

The whole body of the fish was fixed in 10% formalin for at least 48 h and then processed by the method outlined by Bhatia et al. (2013) for histological investigation. The tissues were dehydrated in a graded series of ethanol and xylene using an automated tissue processor (Leica) and then embedded in paraffin using a paraffin-embedding centre (Leica). Rough vertical sectioning of the block was done on a rotary microtome (Microm International). The sections were floated on a water bath at 40°C, lifted on a slide and immediately analysed for the appearance of gonadal cells. Once the gonadal cells began to appear, six sections (4 – 5 µm) were cut. The sections were laid on a glass slide, dried and hydrated in a graded series of ethanol. The sections were stained with haematoxylin and eosin phloxine (HD Scientific), photographed using an Olympus BX51 microscope and analysed with AnalySIS software (Olympus Soft Imaging Solutions GmbH 5.0 build 1235).

The germ cells in the ovaries were identified on the basis of their size and the presence of cortical alveoli or yolk vesicles as outlined by Bhatia et al. (2013) and classified as follows:

a) Perinucleolar oocytes had a large nucleus and homogenously staining, dark ooplasm. Cortical alveoli and yolk vesicles were absent;
b) Cortical alveolar oocytes were larger than perinucleolar oocytes. The cortical alveoli were arranged in a ring near the periphery of the oocytes;
c) Early vitellogenic oocytes showed a beginning of the appearance of yolk vesicles in the centre. The cytoplasm was filled with cortical alveoli and

d) Late vitellogenic oocytes had their entire cytoplasm filled with yolk vesicles and the cortical alveoli were pushed to the periphery.

The developmental stage of the ovaries was classified as follows:

a) Stage 0 (immature): Only perinucleolar oocytes were present in this stage;

b) Stage I (previtellogenic): Abundant perinucleolar oocytes and a few cortical alveolar oocytes were present;

c) Stage II (vitellogenic): early vitellogenic oocytes can be seen in this stage. In addition, some perinucleolar and cortical alveolar oocytes are also present and

d) Stage III (mature): Abundant late vitellogenic oocytes with accumulated vitellogenic granules were present.

The testicular germ cells were classified by the method of Bhatia et al. (2014) as follows:

a) Spermatogonia were large cells with eosinophilic cytoplasm arranged in groups of 3 – 4 near but not limited to the periphery of the testes;

b) Spermatocytes had moderate amount of dark staining cytoplasm. These were arranged in clusters called the spermatocysts throughout the length of the testes;

c) Spermatids were small cells with dense cytoplasm and were found in between the spermatocysts and

d) Spermatozoa were mature germ cells scattered in the tubular lumen. These were the smallest in size with minimal cytoplasm.

The development of the testes was classified into the following stages:

a) Stage 0 (immature): The testes consisted of spermatogonia and spermtaocytes only. No spermatozoa were present in this stage;

b) Stage I (early-spermatogenic): spermatozoa begin to appear in this stage. Abundant spermatocytes were present;

c) Stage II (mid-spermatogenic): Approximately similar proportions of spermatocytes, spermatids and spermatozoa were present and

d) Stage III (mature): All types of germ cells were present. However, the proportion of spermatozoa was the highest.

Hormone analyses

The concentrations of sex steroid hormones - E2 and 11-keto testosterone (11-KT) in the whole-body homogenates were measured using enzyme immunoassay (EIA) kits (Cayman Chemicals) Bhatia et al. (2014c). The method was validated by investigating parallelism between E2 or 11-KT standards and the hormone concentrations in serially diluted (1:2 – 1:256) whole-body homogenates of fish. The dilution of the sample corresponding to 50% bound antibody was used as the dilution factor for the test samples (2 times here). The snap-frozen whole fish bodies were thawed on ice, weighed and homogenised in 1:10 (w/v) homogenisation buffer (pH 7.4) (100 mM KCl, 19 mM KH_2PO_4, 0.02 M HEPES, 1 mM EDTA and 1 mM dithioreitol) using 1 mm zirconium oxide beads (Thermo Fischer Scientific) on Fastprep-24 (MP-Biomedicals) at 6 m/s for 60 sec. The homogenate was centrifuged at 1500 *g* for 60 min at 4°C. The homogenate was separated from the pellet and diluted two times in phosphate buffer saline (pH 7.4).

The wells of the 96-well plate used were coated with anti-rabbit IgG antibody. The E2 standards (6.6 pg/mL – 4000 pg/mL) or 11-KT standards (0.78 pg/mL – 100 pg/mL) were added to wells. Samples (50 µL), in triplicates, were added per well. Hundred microlitres and 50 µL of EIA buffer were added to the wells designated as non-specific binding and

maximum binding (B_0), respectively. E2 (or 11-KT) acetyl cholinesterase (AChE) tracer (50 µL) was added to each well except to the total activity (TA) and the blank (Blk) wells. Then 50 µL E2 (or 11-KT) rabbit anti-serum were added to each well except to the TA, NSB and Blk wells and the plate was incubated at room temperature for 60 min for E2 measurement (or for 18 h at 4 °C for 11-KT measurement). After incubation, the wells were washed five times with wash buffer to remove unbound reagents. Ellman's reagent (200 µL) was added to each well. At this stage 5 µL of the E2 (or 11-KT) tracer was added to the TA wells and the plate was placed on an orbital shaker for 60 – 90 min for E2 measurement (or 90 – 120 min for 11-KT measurement). The luminescence was recorded at 405 nm using a luminometer (Thermo Labsystems multiskan ascent microplate photometer). The intensity of the yellow colour was inversely proportional to the amount of steroid in the sample.

The average absorbance reading of the NSB was subtracted from the average absorbance of B_0 to obtain the corrected B_0. The average NSB absorbance was subtracted from the average sample/standard absorbance and was divided by the corrected $B_0$to obtain B/B_0 (sample or standard bound/maximum bound). The $\%B/B_0$ for the standards was plotted against the standard concentration using linear (y) and log (x) axes and a four-parameter logistic fit was performed. The concentration of the hormones in the samples was determined by reading the value corresponding to $\% B/B_0$ on the x-axis.

Data analyses

Statistical analyses were performed using SigmaPlot 12.5. Four replicates containing 2 fish in each were used for hormone analyses and for histological investigation for each treatment in the present study. There was no significant difference in the values of hormones between the fish across different tanks within the same treatment which confirmed the

absence of tank effects. Hence, the number of replicates used in statistical analyses was eight. Normality of the data was confirmed with Shapiro-Wilk test. The data that passed the normality were subjected to one-way analysis of variance (ANOVA) to determine differences between the treatments. Significance was set at $p \leq 0.05$. The Holm-Sidak test was used to determine significant differences ($p \leq 0.05$) between the groups.

RESULTS

Water quality and physical conditions during the test

During the entire duration of the exposure, temperature was maintained at 23 ± 0.1°C. The conductivity of water in the testing tanks ranged between 1231 – 1241 µS/cm and the pH varied in the normal range of 6.8 – 7.1. The DO levels in the water were above 80% (Supplementary table).

Analytical chemistry

The average of the measured concentrations for the nominal levels of 5, 15 and 50 µg/L of DnBP before the start of the exposure were 5 ± 0.5, 15 ± 1 and 50 ± 6 µg/L, respectively. After 24 h of spiking and exposure, 70 – 80% of the measured concentration of DnBP was still present in the 15 and 50 µg/L treatments. However, in the 5 µg/L treatment, 120% of the measured levels of DnBP were recovered after 24 h. The LOQ and the LOD for the method used to measure DnBP were 0.012 µg/L and 0.004 µg/L, respectively. DnBP levels in the water and solvent control tanks were below LOD before the start of the exposure and below LOQ 24 h after spiking of the test solutions (Supplementary Table).

Mortality and vital indices

After 90 days of exposure, no mortality was found in the juvenile fish suggesting no effect of long-term exposures to environmentally relevant concentrations of DnBP on the survival of fish. The control fish showed normal growth pattern over 90 days of the experiment with significant increases in lengths and weights at each sampling time (Supplementary figure, Figure 1A). Concentration of 5 µg/L of DnBP did not affect the lengths or weights significantly after 30 days. Exposure to 15 and 50 µg/L of DnBP resulted in significant reductions in the weights of the fish with respect to the corresponding controls ($p \leq 0.05$) at all sampling times (Figures 1B). Significant reductions in the lengths of the fish were noted after treatment with DnBP for 60 days at all concentrations ($p \leq 0.05$). The lowest observed effective concentration (LOEC) to induce significant changes in CF was 5 µg/L after 90 days ($p \leq 0.05$) (Figure 1C, Table 1).

Gonadal histopathology

The ovaries of the water and solvent control fish on days 0, 30 and 60 contained only perinucleolar oocytes and were in stage 0 (immature) of development (Figure 2A, 2B. 2C). On day 90, cortical alveolar and early vitellogenic oocytes were also present and the ovaries were in stage II (vitellogenic) of development (Figure 2D). The testes in the water and solvent control fish on days 0 and 30 contained spermatogonia and spermatocytes and were in stage 0 (immature) (Figure 3A, 3B). A few spermatozoa, in addition to the spermatogonia and spermatocytes were seen in the testes after 60 days in the controls (stage I early-spermatogenic) (Figure 3C). After 90 days of the test, approximately similar proportions of all types of germ cells were present in the testes, which were then in stage II (mid-spermatogenic) (Figure 3D). The histological analysis of the gonads in water and solvent controls further suggested that females mature earlier than males. Only 25% of the fish were

males on days 0 and 30. Thirty-three percent males were found after 60 days and their proportion rose to 50% by day 90.

Exposures to 5 µg/L of DnBP for up to 60 d did not have profound effects on gonadal differentiation. Twelve percent of the fish were males containing only spermatogonia and spermatocytes (stage 0). Testes, where found, were immature and vacuolated (Figure 4A). Histological investigation revealed that all the fish exposed to 5 µg/L for 90 days contained ovaries (complete feminisation). Similar results were noted in fish exposed to 50 µg/L of DnBP for 30 and 60 days. However, the ovaries from DnBP-treated fish were immature (stage 0) even after 60 days of the test (Figures 4B, 4C). Intersex condition was noted in 50% of the fish exposed to 50 µg/L DnBP on day 90 (Figure 4D). Interestingly, similar histological results were noted in 15 µg/L treated fish. Ovaries in all DnBP-treated fish were regressed and immature containing only perinucleolar oocytes. Absence of vitellogenic oocytes confirmed that the ovaries were immature.

Hormone analyses

In control fish, the whole-body concentrations of E2 did not differ significantly after days 30, 60 and 90 of the test and were significantly lower than those in the 0 day fish ($p \leq 0.05$). Similar trend was seen in the 11-KT concentrations. Exposures to 5 µg/L of DnBP for up to 60 days did not result in significant change in the E2 or 11-KT levels. However, on day 90, a significant increase in E2 and decrease in 11-KT levels were noted in this treatment ($p \leq 0.05$). Treatment with 15 µg/L DnBP induced E2 and 11-KT levels at all time intervals investigated with respect to the corresponding control fish. Within the 15 µg/L treatment, the increase in E2 was significant only after 90 days of exposure ($p \leq 0.05$). In the 50 µg/L of DnBP treated fish, significant increase in the E2 and 11-KT concentrations were noted only

on day 60 ($p \leq 0.05$) (Figures 5A, 5B). Significant inductions in the E2/11-KT levels were measured in fish exposed to 5 µg/L of DnBP for 90 days and in those exposed to 50 µg/L DnBP for 30 days (Figure 5C).

Based on the data obtained on day 90 in the present study, the LOEC of DnBP to cause mortality in fish is > 50 µg/L. The LOEC to significantly affect the length, weight and CF of the fish is 5 µg/L. 100% feminisation of the gonad is at 5 µg/L while inter-sex condition can be seen at LOEC of 50 µg/L of DnBP. The LOEC for whole-body E2, 11-KT and E2/11-KT changes is 5 µg/L on day 90 of exposure (Table 1).

DISUSSION

The aim of the present study was to investigate the effects of long-term exposures to environmentally relevant concentrations of DnBP on gonadal differentiation and the whole-body concentrations of sex steroid hormones in juvenile Murray rainbowfish. We found complete feminisation of the gonads after treatment of fish with 15 and 50 µg/L DnBP for 30 or 60 days.

Our data are consistent with the earlier findings reporting inhibitory effects of phthalates on the growth of fish. Juvenile guppies treated with 10 µg/L DEHP for 91 days showed 40% and 70% reductions in their lengths and weights, respectively (Zanotelli *et al.,* 2010). Exposure of <3 day old Japanese medaka to 502 µg/L DEHP for 168 days has been reported to result in 13% weight reduction (Defoe *et al.,* 1990). Similar reductions in weights have been reported in medaka at 5 months, after treating eggs until hatch (Chikae *et al.,* 2004) or fry for 3 wk (Chikae *et al.,* 2004) with 10 µg/L DEHP. Condition factor, based on lengths and weights, has been regarded as a reliable tool to determine fish health and welfare

(Bervoets and Blust, 2003; Bolger and Connolly 1989). High CF on day 90 after exposures to 5 – 50 µg/L DnBP suggest adverse affects on the general health of fish. This suggests that low concentrations of phthalates delay the developmental process in fish without affecting their general health (Zanotelli *et al.,* 2010).

Although histological investigation revealed complete feminisation of the gonads after treatments with 5 µg/L of DnBP for 90 days and with 15 and 50 µg/L of DnBP at all sampling times, the ovaries were regressed, smaller in size and contained only perinucleolar oocytes. These observations confirm the earlier findings of the adverse effects of treatment with phthalates on the development of oocytes in fish. The oocytes failed to mature when medaka were exposed to 10 µg/L DEHP for 90 day (Kim *et al.,* 2002) or to 40 µg/L DEHP for 21 days (Carnevali *et al.,* 2010). A reduction in the sizes of mature oocytes was reported in mussels (*Mytilus edulis*) treated with 50 µg/L of diallyl phthalate for 21 days (Aarab *et al.,* 2006). In our recent study we treated adult Murray rainbowfish with 125 – 1000 µg/L DnBP for 7 days and found reductions in the sizes of early vitellogenic oocytes and increase in the sizes of perinucleolar and cortical alveolar oocytes (Bhatia *et al.,* 2013). These findings align with an *in vitro* study where oocytes of zebrafish co-cultured with DEHP failed to undergo germinal vesicle breakdown (GVBD) (Tokumoto *et al.,* 2005). Exposure of marine medaka (*Oryzias melastigma*) for 6 months decreased the egg production of exposed females (Ye *et al.,* 2014).

The failure of the oocytes to mature after treatment with DnBP could be due to low VTG concentrations or the failure to incorporate VTG into the growing oocytes. Although we did not measure VTG concentration in the present study, the absence of vitellogenic oocytes in DnBP-treated fish supports our hypothesis. In our previous study, we had reported

significant reductions in the circulating levels of VTG in female rainbowfish after DnBP exposure (Bhatia *et al.,* 2013). Vitellogenin is synthesised in the liver of egg-laying animals in response to estrogens that bind to the ERs in liver and activate the transcription of the gene for VTG. Interestingly, VTG changes in fish are sex-specific. Previous studies have reported induction of VTG protein after exposure to phthalates in male carp (Barse *et al.,* 2007) and in male Murray rainbowfish (Bhatia *et al.,* 2014). The VTG levels decrease after exposure to phthalates in females of the fish species as reported in Murray rainbowfish (Bhatia *et al.,* 2013) and medaka (Kim *et al.,* 2002). Further research into VTG changes and molecular mechanisms underlying oocyte-development after exposure to phthalates in juvenile fish are warranted.

Studies have also reported deleterious effects of exposure to phthalates on the development of germ cells in testes in fish. Treatment of zebrafish with a high concentration (500 mg/kg body weight) of DEHP for 10 days resulted in a significant decline in the proportion of spermatocytes (Uren-Webster *et al.,* 2010). We exposed adult Murray rainbowfish to 125 – 1000 µg/L DnBP for 7 days and observed a significant reduction in the proportion of spermatozoa. This was accompanied by an increase in the germinal epithelium height (Bhatia *et al.,* 2014). Few mammalian studies have attempted to elucidate the mechanism of action of phthalates in inhibiting germ cell development in the testis. *In vitro* culture of the Sertoli cells with monoethylhexyl phthalate (MEHP) inhibited the follicle stimulating hormone receptor-adenyl cyclase system (Mahvi *et al.,* 2005), thereby, inhibiting initiation of spermatogenesis by FSH in rats (Grasso *et al.,* 1993; Lloyd and Foster 1988). Mixture of phthalates with one another and with other anti-androgens resulted in dose-additive disruption of the Leydig and the Sertoli cells maturation leading to reduction in the proportion of the germ cells in rats (Howdeshell *et al.,* 2008). Phthalates have also been

reported to disrupt spermatogenesis in medaka and zebrafish by inducing the expression of the genes encoding peroxisome proliferator-activator receptors (PPAR) (Chikae *et al.,* 2004; Uren-Webster *et al.,* 2010). The molecular mechanism involved in the hindrance to spermatogenesis caused by phthalates needs to be investigated in Murray rainbowfish.

Interestingly, the effects of DnBP on testes development seen in the present study are consistent with the findings in fish exposed to estrogens like E2, EE2 and 4-nonylphenol (Harries *et al.,* 2000; Miles-Richardson *et al.,* 1999; Pawlowski *et al.,* 2004). We believe the absence of testicular germ cells and the development of oocytes in the gonads could be due to high levels of E2 measured in the present study. Treatment of male Japanese frog (*Rana rugosa*) to 280 µg/L DnBP during gonadal differentiation (19 – 23 dph) induced partial ovarian development in the testes (Ohtani *et al.,* 2000). Ovo-testis has been reported in pikeperch exposed to up to 2 g DnBP during sex differentiation period (61 – 96 dph) (Jarmolowicz *et al.,* 2013). Intersex has also been reported in Atlantic salmon (*Salmo salar*) exposed to 1.5 g/kg DEHP (Norman *et al.,* 2007). However, the concentrations of phthalates used in these studies were either unrealistically high or the route of exposure was diet.

The effects of phthalates on the hormonal profile appear to be gender-specific in fish. In addition, the concentrations of the phthalates used explicit confounding results. Exposure of marine medaka for 6 months to 500 µg/L DEHP resulted in increase in E2 concentrations in males as well as in females while testosterone (T) levels were higher only in females (Ye *et al.,* 2014). Treatment of Chinese rare minnow (*Gobiocypris rarus*) to >40 µg/L DEHP for 21 days led to an increase in the T and E2 levels (along with up-regulation of *cyp19a*) in males (Wang *et al.,* 2013). Aoki and co-workers reported an induction in the levels of T in the plasma of male three-spined stickleback after exposures to up to 35 µg/L of DnBP for 22

days. However, no significant change was reported in the 11-KT levels (Aoki *et al.,* 2011). It should be emphasised that in these studies, the circulating levels of the hormones in the plasma were measured. However, we measured the whole body concentrations of E2 and 11-KT.

Phthalates have been reported to displace E2 from ERs in rainbow trout (Matthews *et al.,* 2000) and in rats (Blair *et al.,* 2000). The increase in the concentration of E2 measured in the present study could be due to the displacement of E2 from ERs or due to the up-regulation of the genes encoding ERs and the consequent activation of CYP19A1, the key enzyme catalysing the conversion of T to E2 after treatment with DnBP (Bhatia *et al.,* 2014; Callard *et al.,* 2001; Wang *et al.,* 2013). In addition, phthalates like DEHP and MEHP have been demonstrated to up-regulate the expression of the gene encoding nuclear receptor subfamily-5 group a member 2 (nr5a2) in marine medaka (Ye *et al.,* 2014) which has been reported to activate aromatase in American bullfrog (*Rana catesbeiana*) (Mayer *et al.,* 2002).

Aoki and co-workers reported increase in the testosterone concentrations in three-spined stickleback after exposure to 22 and 35 µg/L of DnBP (Aoki *et al.,* 2011). Testosterone is the precursor of 11-KT and E2. Although, the testosterone levels were not measured in the present study, we believe an increase in the testosterone levels could have resulted in high 11-KT concentrations. However, these findings need investigation and validation in Murray rainbowfish. We strongly recommend long-term exposures investigating the expression levels of the genes involved in genetic sex-determination like *Dmrt1*, *Dmy* (testicular differentiation) and *cyp19a*, *Foxl2* (ovarian differentiation) can help better understand the incidences of sex reversal or intersex gonads in sexually undifferentiated fish.

The data presented here provide new insight into the endocrine disruption caused after exposures to environmentally relevant concentrations of phthalates in sexually undifferentiated Murray rainbowfish. Estrogenicity, as divulged from the E2/11-KT values, was induced after exposures to 5 µg/L of DnBP for 90 days and 50 µg/L of DnBP for 30 days. The CF was adversely affected only 90 days of exposure to 5, 15 and 50 µg/L of DnBP. Treatment with DnBP affected the gonadal differentiation and development with skewed proportions of sex, favouring the females. However, the germ cell development in both ovaries and testes was hindered.

CONCLUSIONS

In conclusion, exposure to 5 µg/L of DnBP for up to 30 days was not deleterious to the general and reproductive health of the juvenile fish. Treatment with 15 and 50 µg/L of DnBP could induce complete feminisation of the gonads with altered E2 and 11-KT levels in 30 days. Although the gonads were completely feminised, the oocyte-development was hindered in DnBP-treated fish. The LOEC values of DnBP for survival, length, weight and body condition factor proposed in the present study suggest the potential population-relevant adverse effects. In addition, the LOEC values for gonadal histology and steroid hormone concentrations can provide mechanistic signals for future chronic testing studies. Currently, Australia recommends maximum of 9.9 µg/L of DnBP for aquatic ecosystem (http://www.environment.gov.au/resource/australian-and-new-zealand-guidelines-fresh-and-marine-water-quality-volume-1-guidelines). We found that the general health, gonadal development and sex-steroid hormone profile of the fish was adversely affected after treatments with low concentrations of DnBP (5 µg/L) for longer periods (90 days). We believe that although the levels of DnBP detected in Australian environment are safe, the water-quality guidelines are not protective for Murray rainbowfish. Since the exposure of

fresh-water fish populations to DnBP in the aquatic environment is continuous, there is a need to conduct full life-cycle test and calculate adverse NOEC and predicted no-effect concentrations (PNEC) values.

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References

Aarab N, Lemaire-Gony S, Unruh E, Hansen PD, Larsen BK, Andersen OK, Narbonne JR. 2006. Preliminary study of responses in mussel (Mytilus edilus) exposed to bisphenol A, diallyl phthalate and tetrabromodiphenyl ether. *Aquat. Toxicol.* **78**:S86-S92. 10.1016/j.aquatox.2006.02.021:

Aoki KAA, Harris CA, Katsiadaki I, Sumpter JP. 2011. Evidence suggesting that di-n-butyl phthalate has antiandrogenic effects in fish. *Environ. Toxicol. Chem.* **30**:1338-1345. 10.1002/etc.502:

Australia Government Department of Environment Website (http://www.environment.gov.au/resource/australian-and-new-zealand-guidelines-fresh-and-marine-water-quality-volume-1-guidelines) Accessed June 1, 2014.

Autian J. 1973. Toxicity and health threats of phthalate esters: review of the literature. *Environ. Health Persp.*3-26.

Barse AV, Chakrabarti T, Ghosh TK, Pal AK, Jadhao SB. 2007. Endocrine disruption and metabolic changes following exposure of Cyprinus carpio to diethyl phthalate. *Pest. Biochem. Physiol.*. **88**:36-42. 10.1016/j.pestbp.2006.08.009:

Bhatia H, Kumar A, Du J, Chapman J, McLaughlin MJ. 2013. Di-n-butyl phthalate causes antiestrogenic effects in female murray rainbowfish (*Melanotaenia fluviatilis*). *Environ. Toxicol. Chem.* **32**:2335-44. 10.1002/etc.2304:

Bhatia H, Kumar A, Ogino Y, Du J, Gregg A, Chapman J, McLaughlin MJ, Iguchi T. 2014a. Effects of the commercial anti-androgen, flutamide on the biomarkers of reproduction in male Murray rainbowfish (*Melanotaenia fluviatilis*). *Environ Toxicol Chem*. 10.1002/etc.2524:

Bhatia H, Kumar A, Ogino Y, Gregg A, Chapman J, McLaughlin MJ, Iguchi T. 2014b. Di-n-butyl phthalate causes estrogenic effects in adult male Murray rainbowfish

(*Melanotaenia fluviatilis*). *Aquat. Toxicol*. **149C**:103-115. 10.1016/j.aquatox.2014.01.025:

Bhatia H, Kumar A, Chapman JC, McLaughlin M. 2014c. Effects of short-term exposure to the model anti-androgen, flutamide on reproductive function based endpoints in female Murray rainbowfish (*Melanotaenia fluviatilis*). *Ecotoxicol. Environ. Safety* 10.1016/j.ecoenv.2014.07.027

Bervoets, L., Blust, R., 2003. Metal concentrations in water, sediment and gudgeon (*Gobio gobio*) from a pollution gradient:relationship with fish condition factor. *Environ.Pollut*.126,9–19.

Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, Tong WD, Shi LM, Perkins R, Sheehan DM. 2000. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands. *Toxicol. Sci.* **54**:138-153. 10.1093/toxsci/54.1.138:

Bolger, T. And Connolly, P. L., 1989. The selection of suitable indexes for the measurement and analysis of fish condition. *J. Fish Biol.* 34: 171-182.

Callard GV, Tchoudakova AV, Kishida M, Wood E. 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. *J. Steroid Biochem. Mol. Biol.* **79**:305-314. Pii s0960-0760(01)00147-910.1016/s0960-0760(01)00147-9:

Carnevali O, Tosti L, Speciale C, Peng C, Zhu Y, Maradonna F. 2010. DEHP Impairs Zebrafish Reproduction by Affecting Critical Factors in Oogenesis. *Plos One*. **5** e1020110.1371/journal.pone.0010201:

Chikae M, Hatano Y, Ikeda R, Morita Y, Hasan Q, Tamiya E. 2004. Effects of bis(2-ethylhexyl) phthalate and benzo a pyrene on the embryos of Japanese medaka

(*Oryzias latipes*). *Environ. Toxicol. Pharmacol.* **16**:141-145. 10.1016/j.etap.2003.11.007:

Chikae M, Ikeda R, Hatano Y, Hasan Q, Morita Y, Tamiya E. 2004. Effects of bis(2-ethylhexyl) phthalate, gamma-hexachlorocyclohexane, and 17 beta-estradiol on the fry stage of medaka (*Oryzias latipes*). *Environ. Toxicol. Pharmacol.* **18**:9-12. 10.1016/j.etap.2004.04.004:

Clara M, Windhofer G, Hartl W, Braun K, Simon M, Gans O, Scheffknecht C, Chovanec A. 2010. Occurrence of phthalates in surface runoff, untreated and treated wastewater and fate during wastewater treatment. *Chemosphere*. **78**:1078-1084. 10.1016/j.chemosphere.2009.12.052:

Defoe DL, Holcombe GW, Hammermeister DE, Biesinger KE. 1990. Solubility and toxicityt of 8 phthalate esters to 4 aquatic organisms. *Environ. Toxicol. Chem.* **9**:623-636. 10.1897/1552-8618(1990)9[623:satoep]2.0.co;2:

Fatoki OS, Bornman M, Ravandhalala L, Chimuka L, Genthe B, Adeniyi A. 2010. Phthalate ester plasticizers in freshwater systems of Venda, South Africa and potential health effects. *Water SA*. **36**:117-125.

Fatoki OS, Vernon F. 1990. Phthalate-esters in rivers of the greater Manchester area, UK. *Sci. Total Environ.* **95**:227-232. 10.1016/0048-9697(90)90067-5:

Foster PMD, Mylchreest E, Gaido KW, Sar M. 2001. Effects of phthalate esters on the developing reproductive tract of male rats. *Apmis*. **109**:S272-S276.

Fromme H, Kuchler T, Otto T, Pilz K, Muller J, Wenzel A. 2002. Occurrence of phthalates and bisphenol A and F in the environment. *Water Research*. **36**:1429-1438. Pii s0043-1354(01)00367-010.1016/s0043-1354(01)00367-0:

Grasso P, Heindel JJ, Powell CJ, Reichert LE. 1993. Effects of mono(2-ethylhexyl) phthalate, a testicular toxicant, on follicle-stimulating-hormone binding to

membranes from cultured rat sertoli cells. *Biol. Reprod.* **48**:454-459. 10.1095/biolreprod48.3.454:

Gray MA, Metcalfe CD. 1997. Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to p-nonylphenol. *Environ. Toxicol. Chem.* **16**:1082-1086. 10.1897/1551 5028(1997)016<1082:iotoij>2.3.co;2:

Han ZX, Lv CX, Li H. 2009. Effects of Bis(2-ethylhexyl) Phthalate on Sex Hormones of Common Carp (*Cyprinus carpio*) and the Protection of Zinc. *Synthesis and Reactivity in Inorganic Metal-Organic and Nano-Metal Chemistry*. **39**:100-105. Pii 90931948310.1080/15533170902772867:

Harries JE, Runnalls T, Hill E, Harris CA, Maddix S, Sumpter JP, Tyler CR. 2000. Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). *Environ. Sci. Technol.* **34**:3003-3011. 10.1021/es991292a:

Harris CA, Henttu P, Parker MG, Sumpter JP. 1997. The estrogenic activity of phthalate esters in vitro. *Environ. Health Persp.* **105**:802-811. 10.1289/ehp.97105802:

Howdeshell KL, Rider CV, Wilson VS, Gray LE, Jr. 2008. Mechanisms of action of phthalate esters, individually and in combination, to induce abnormal reproductive development in male laboratory rats. *Environ. Res.* **108**:168-176. 10.1016/j.envres.2008.08.009:

Jarmolowicz S, Demska-Zakes K, Zakes Z. 2013. Impact of di-n-butyl phthalate on reproductive system development in European pikeperch (*Sander lucioperca*). *Acta Veterinaria Brno*. **82**:197-201. 10.2754/avb201382020197:

Jobling S, Reynolds T, White R, Parker MG, Sumpter J. 1995. A variety of environmentally persistent chemicals, including some phthalate plasticizers are weakly estrogenic. *Environ. Health Persp*. **103**:582-587.

Kim EJ, Kim JW, Lee SK. 2002. Inhibition of oocyte development in Japanese medaka (*Oryzias latipes*) exposed to di-2-ethylhexyl phthalate. *Environ. Int.* **28**:359-365. Pii s0160-4120(02)00058-210.1016/s0160-4120(02)00058-2:

Kim HS, Kim TS, Shin JH, Moon HJ, Kang IH, Kim IY, Oh JY, Han SY. 2004. Neonatal exposure to di(n-butyl) phthalate (DBP) alters male reproductive-tract development. *J. Toxicol. Environ. Health.* **67**:2045-2060. 10.1080/15287390490514859:

Lloyd SC, Foster PMD. 1988. Effect of mono-(2-ethylhexyl)phthalate on follicle-stimulating hormone responsiveness of cultured rat Sertoli cells. *Toxicol. App. Pharmacol.* **95**:484-489. http://dx.doi.org/10.1016/0041-008X(88)90366-3:

Mahvi AH, Bazrafshan E, Jahed GR. 2005. Evaluation of COD determination by ISO 6060 method, comparing with standard method (5220, B). *Pak. J. Biol. Sci.* **8**:892-894.

Matthews J, Celius T, Halgren R, Zacharewski T. 2000. Differential estrogen receptor binding of estrogenic substances: a species comparison. *J Steroid Biochem. Mol. Biol.* **74**:223-234. 10.1016/s0960-0760(00)00126-6:

Mayer LP, Overstreet SL, Dyer CA, Propper CR. 2002. Sexually dimorphic expression of steroidogenic factor 1 (SF-1) in developing gonads of the American bullfrog, *Rana catesbeiana*. *Gen. Comp. Endocrinol.* **127**:40-47. 10.1016/s0016-6480(02)00019-9:

Miles-Richardson SR, Kramer VJ, Fitzgerald SD, Render JA, Yamini B, Barbee SJ, Giesy JP. 1999. Effects of waterborne exposure of 17b-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquat. Toxicol.* **47**:129-145.

Mylchreest E, Wallace DG, Cattley RC, Foster PMD. 2000. Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation. *Toxicol. Sci.* **55**:143-151. 10.1093/toxsci/55.1.143:

Nimrod AC, Benson WH. 1998. Reproduction and development of Japanese medaka following an early life stage exposure to xenoestrogens. *Aquat. Toxicol.* **44**:141-156.

Norman A, Borjeson H, David F, Tienpont B, Norrgren L. 2007. Studies of uptake, elimination, and late effects in atlantic salmon (*Salmo salar*) dietary exposed to di-2-ethylhexyl phthalate (DEHP) during early life. *Arch. Environ. Contam. Toxicol.* **52**:235-242. 10.1007/s00244-005-5089-y:

Ohtani H, Miura I, Ichikawa Y. 2000. Effects of dibutyl phthalate as an environmental endocrine disrupter on gonadal sex differentiation of genetic males of the frog *Rana rugosa*. *Environ. Health Persp.* **108**:1189-1193. 10.1289/ehp.001081189:

Pawlowski S, van Aerle R, Tyler CR, Braunbeck T. 2004. Effects of 17 alpha-ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay. *Ecotoxicol. Environ. Safety*. **57**:330-345. 10.1016/j.ecoenv.2003.07.019:

Shen O, Du G, Sun H, Wu W, Jiang Y, Song L, Wang X. 2009. Comparison of *in vitro* hormone activities of selected phthalates using reporter gene assays. *Toxicol. Lett.*. **191**:9-14. 10.1016/j.toxlet.2009.07.019:

Suzuki T, Yaguchi K, Suzuki S, Suga T. 2001. Monitoring of phthalic acid monoesters in river water by solid-phase extraction and GC-MS determination. *Environ. Sci. Technol.* **35**:3757-3763. 10.1021/es001860i:

Tan BLL, Hawker DW, Mueller JF, Leusch FDL, Tremblay LA, Chapman HF. 2007. Modelling of the fate of selected endocrine disruptors in a municipal wastewater treatment plant in South East Queensland, Australia. *Chemosphere*. **69**:644-654. 10.1016/j.chemosphere.2007.02.057:

Tokumoto T, Tokumoto M, Nagahama Y. 2005. Induction and inhibition of oocyte maturation by EDCs in zebrafish. *Reprod. Biol. Endocrinol.* **3** 69 10.1186/1477-7827-3-69:

Uren-Webster TM, Lewis C, Filby AL, Paull GC, Santos EM. 2010. Mechanisms of toxicity of di(2-ethylhexyl) phthalate on the reproductive health of male zebrafish. *Aquat. Toxicol*. **99**:360-369. 10.1016/j.aquatox.2010.05.015:

Vethaak AD, Lahr J, Schrap SM, Belfroid AC, Rijs GBJ, Gerritsen A, de Boer J, Bulder AS, Grinwis GCM, Kuiper RV, Legler J, Murk TAJ, Peijnenburg W, Verhaar HJM, de Voogt P. 2005. An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. *Chemosphere*. **59**:511-524. 10.1016/j.chemosphere.2004.12.053:

Wang X, Yang Y, Zhang L, Ma Y, Han J, Yang L, Zhou B. 2013. Endocrine disruption by di-(2-ethylhexyl)-phthalate in Chinese rare minnow (*Gobiocypris rarus*). *Environ. Toxicol. Chem.* **32**:1846-1854. 10.1002/etc.2261:

Wolf C, Jr., Lambright C, Mann P, Price M, Cooper RL, Ostby J, Gray LE, Jr. 1999. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol. Ind. Health*. **15**:94-118. 10.1177/074823379901500109:

Ye T, Kang M, Huang Q, Fang C, Chen Y, Shen H, Dong S. 2014. Exposure to DEHP and MEHP from hatching to adulthood causes reproductive dysfunction and endocrine disruption in marine medaka (*Oryzias melastigma*). *Aquat. Toxicol.* **146**:115-126.

Zanotelli VRT, Neuhauss SCF, Ehrengruber MU. 2010. Long-term exposure to bis(2-ethylhexyl)phthalate (DEHP) inhibits growth of guppy fish (*Poecilia reticulata*). *J. App. Toxicol.*. **30**:29-33. 10.1002/jat.1468:

Zeng F, Cui K, Xie Z, Liu M, Li Y, Lin Y, Zeng Z, Li F. 2008. Occurrence of phthalate esters in water and sediment of urban lakes in a subtropical city, Guangzhou, South China. *Environ. Int.* **34**:372-380. 10.1016/j.envint.2007.09.002:

Table 1: Lowest observed effective concentrations (LOEC) of di-n-butyl phthalate that can cause physiological effects in juvenile Murray rainbowfish

	30 days	60 days	90 days
Survival of fish	> 50 µg/L	> 50 µg/L	> 50 µg/L
Length of body	> 50 µg/L	5 µg/L	5 µg/L
Weight	15 µg/L	5 µg/L	5 µg/L
Condition factor	> 50 µg/L	> 50 µg/L	5 µg/L
Intersex gonad	> 50 µg/L	> 50 µg/L	50 µg/L
100% Feminisation of gonad	15 µg/L	15 µg/L	5 µg/L
Whole-body E2	15 µg/L	15 µg/L	5 µg/L
Whole-body 11-KT	15 µg/L	15 µg/L	5 µg/L
Whole-body E2/11-KT	50 µg/L	> 50 µg/L	5 µg/L

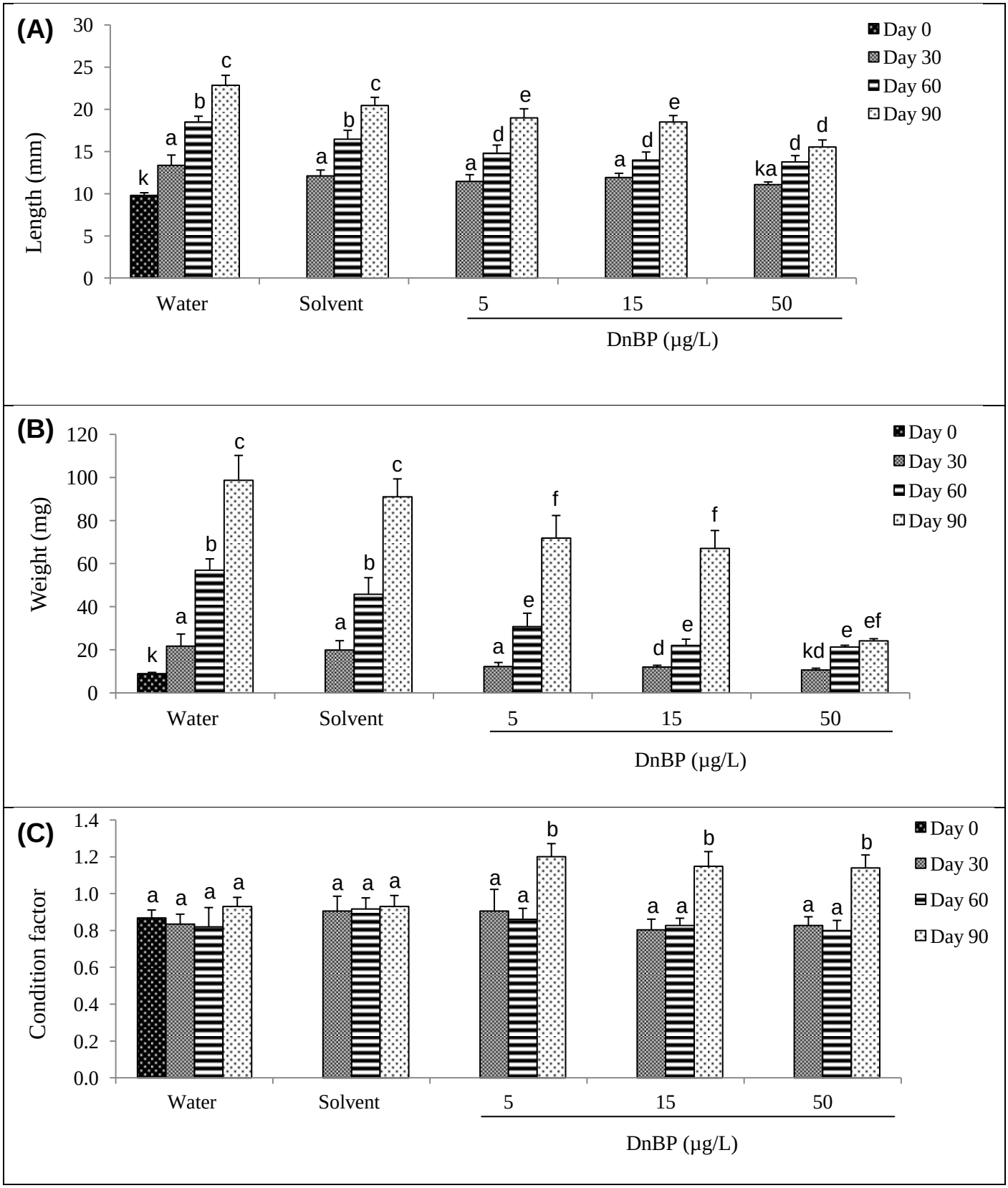


Figure 1: (A) Lengths (B) Weights and (C) Condition factors of juvenile Murray rainbowfish after exposures to 5, 15 and 50 µg/L DnBP for 30, 60 or 90 days each. Bars represent standard error. Bars with different letters are significantly different ($p \leq 0.05$) (n=8).

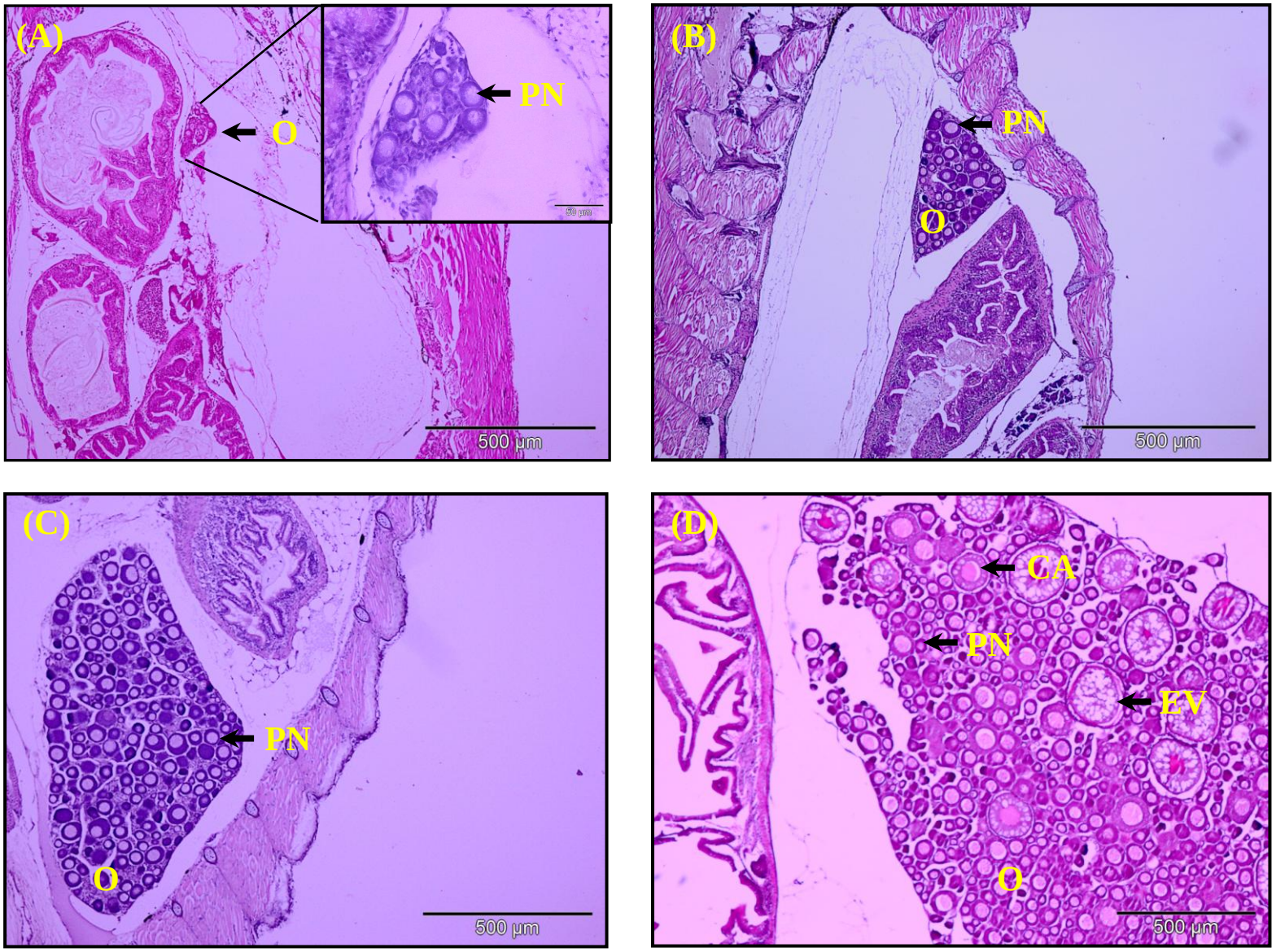
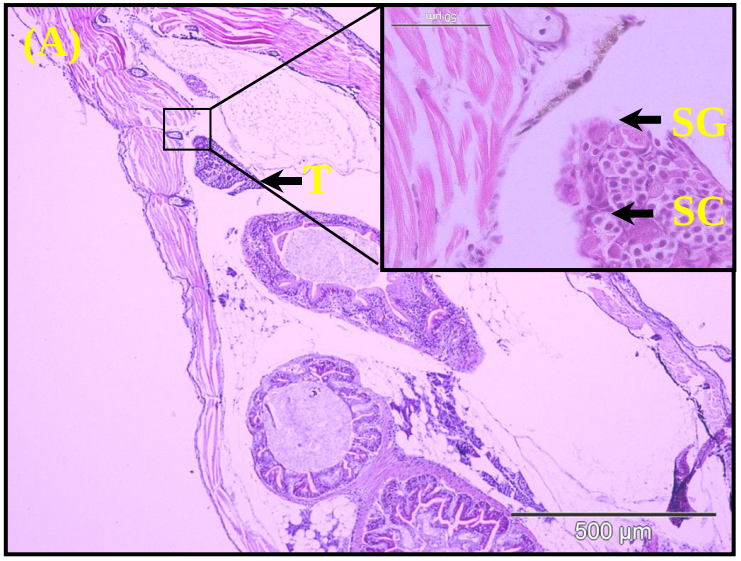
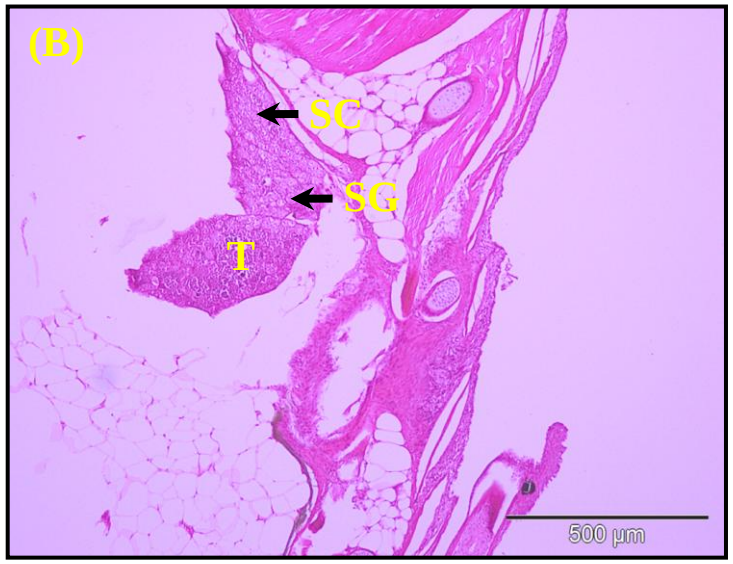

(A)
O
PN
500 µm
(B)
PN
O
500 µm
(C)
PN
O
500 µm
(D)
CA
PN
EV
O
500 µm

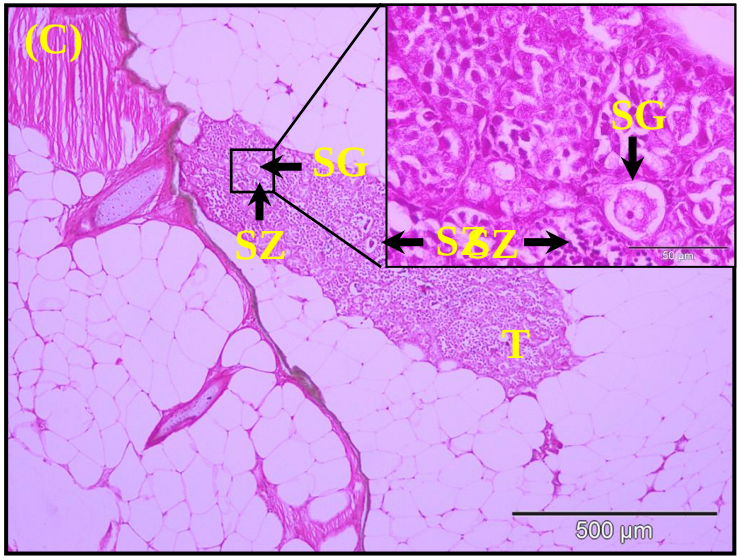
Figure 2: Photomicrographs of the vertical sections of the abdominal region of juvenile Murray rainbowfish from the control group showing the development of the ovaries on (A) day 0 (Stage 0 – immature), (B) day 30 (Stage 0 – immature), (C) day 60 (Stage 0 – immature) and (D) day 90 of the test (Stage II – vitellogenic)

Note the increase in the size of the ovary at each sampling time and the presence of vitellogenic oocytes on day 90.

O: Ovary, PN: Perinucleolar oocytes, CA: Cortical alveolar oocytes, EV: Early vitellogenic oocytes


(A)
SG
T
SC
500 µm


(B)
SC
SG
T
500 µm


(C)
SG
SZ
SG
SZ
T
500 µm

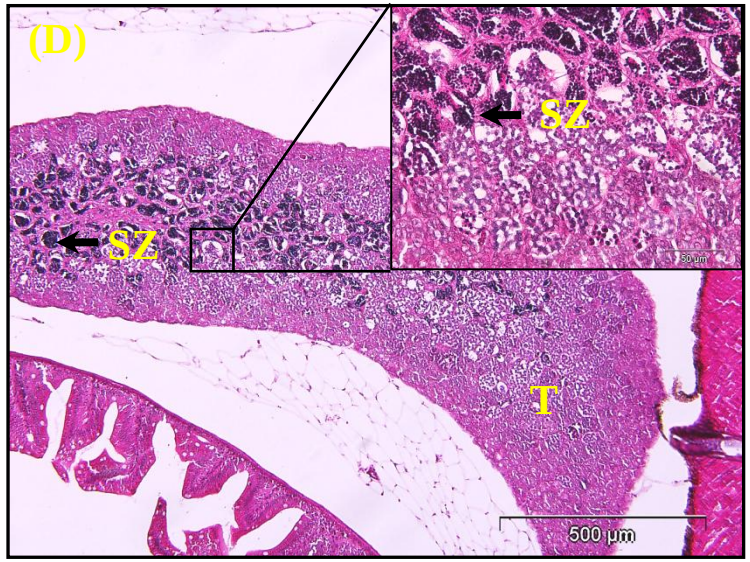

(D)
SZ
SZ
T
500 µm

Figure 3: Photomicrographs of the vertical sections of the abdominal region of juvenile Murray rainbowfish from the control group showing the development of the development of the testes on (A) day 0 (Stage 0 – immature), (B) day 30 (Stage 0 – immature), (C) day 60 (Stage I – early spermatogenic) and (D) day 90 (Stage II – mid-spermatogenic).

T: Testis, SG: Spermatogonia, SC: Spermatocytes, SZ: Spermatozoa

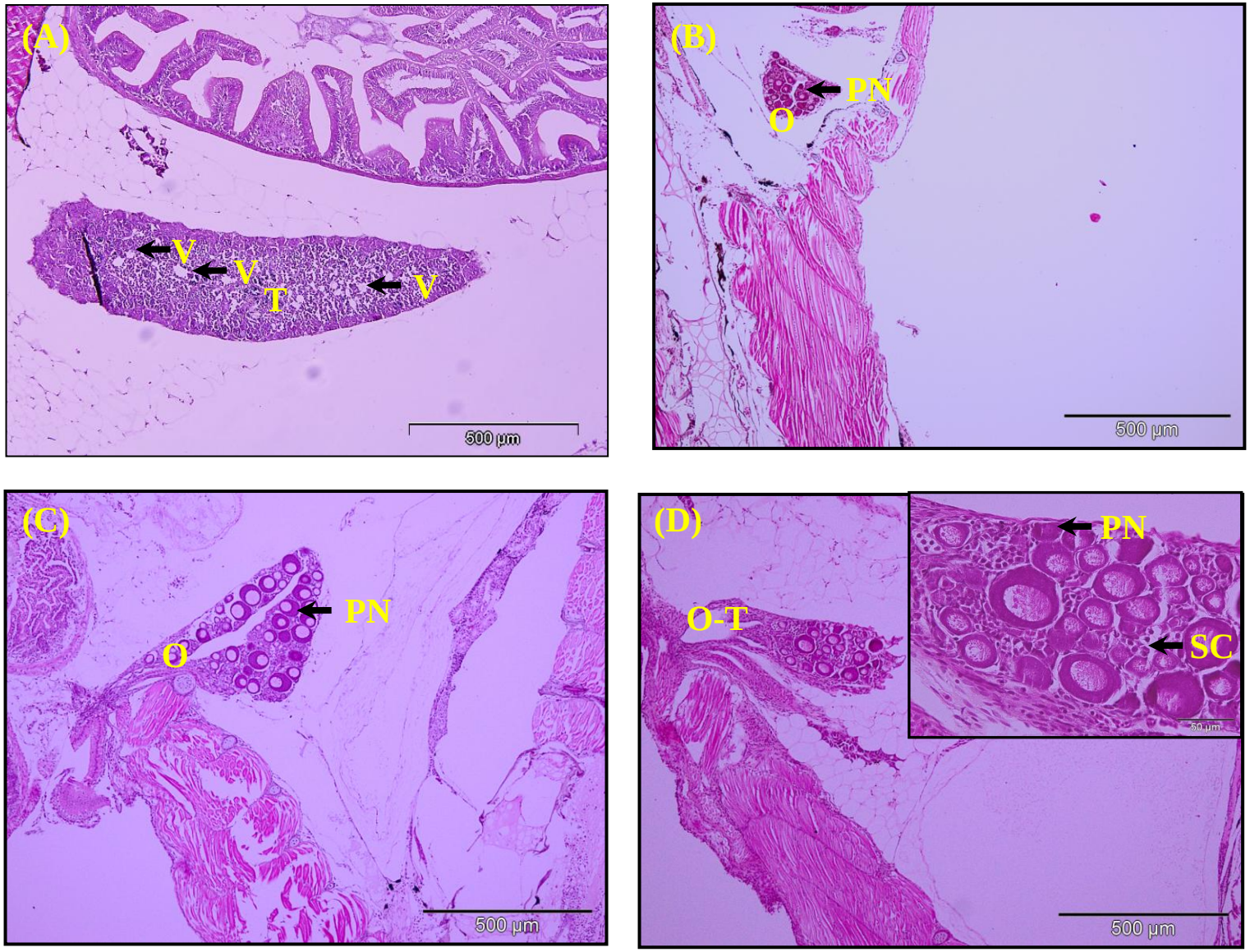

(A)
V
V
T
V
500 µm
(B)
PN
O
500 µm
(C)
PN
O
500 µm
(D)
O-T
PN
SC
500 µm

Figure 4: Photomicrographs of the vertical sections of the abdominal region of juvenile Murray rainbowfish showing the gonads after exposures to (A) 5 µg/L di-n-butyl phthalate (DnBP) for 60 days (Stage 0 – immature); (B) 50 µg/L DnBP for 30 days (Stage 0 – immature), (C) 50 µg/L DnBP for 60 days (Stage 0 – immature) and (D) 50 µg/L DnBP for 90 days (intersex).

Note the reduced proportion of spermatozoa and vacuolation in the testis. Also note the regressed size of the gonad and the absence of vitellogenic oocytes after exposures to 50 µg/L of DnBP.

T: Testis, V: Vacuolation, O: Ovary, O-T: Ovo-testis, PN: Perinucleolar oocytes, SC: Spermatocytes

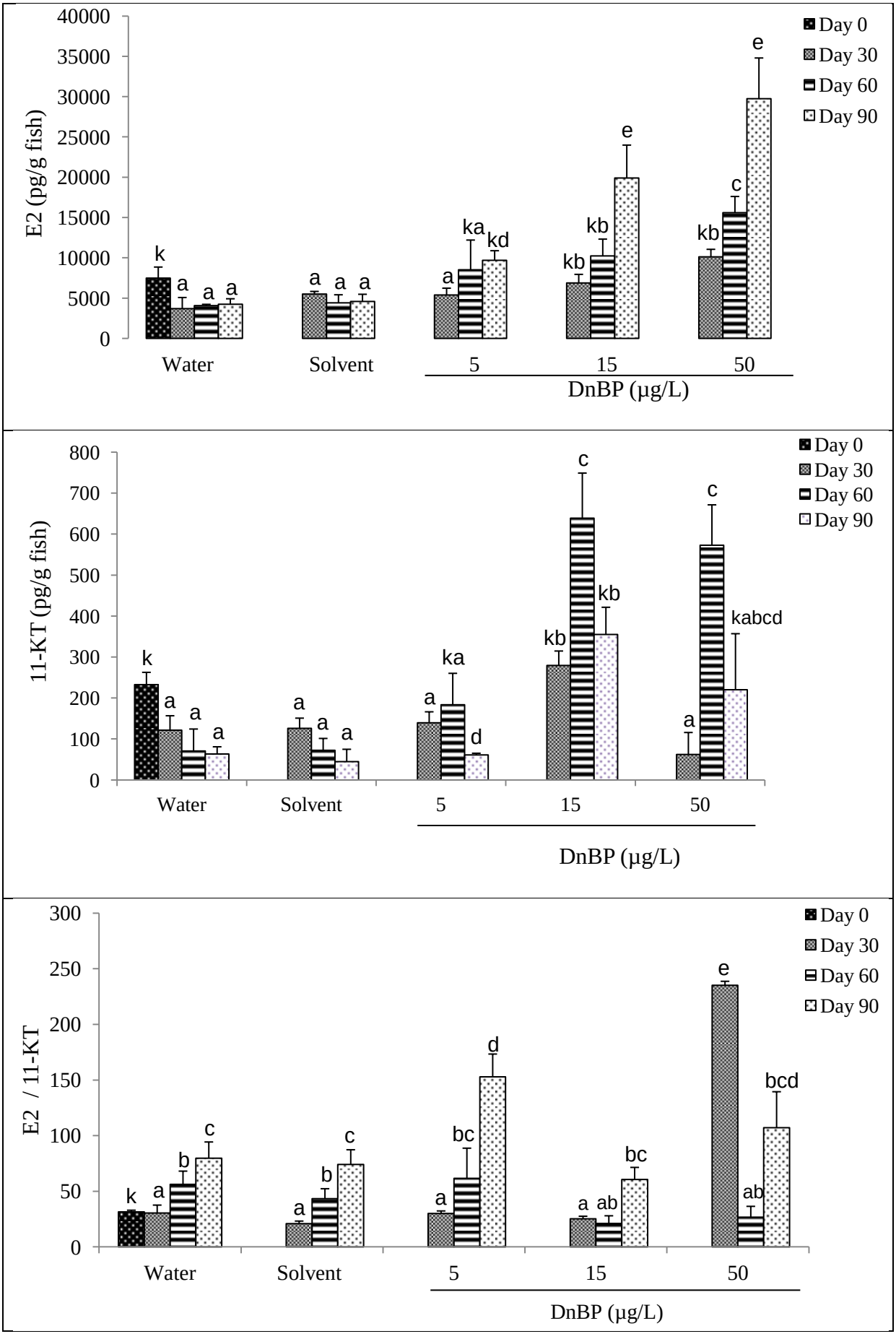
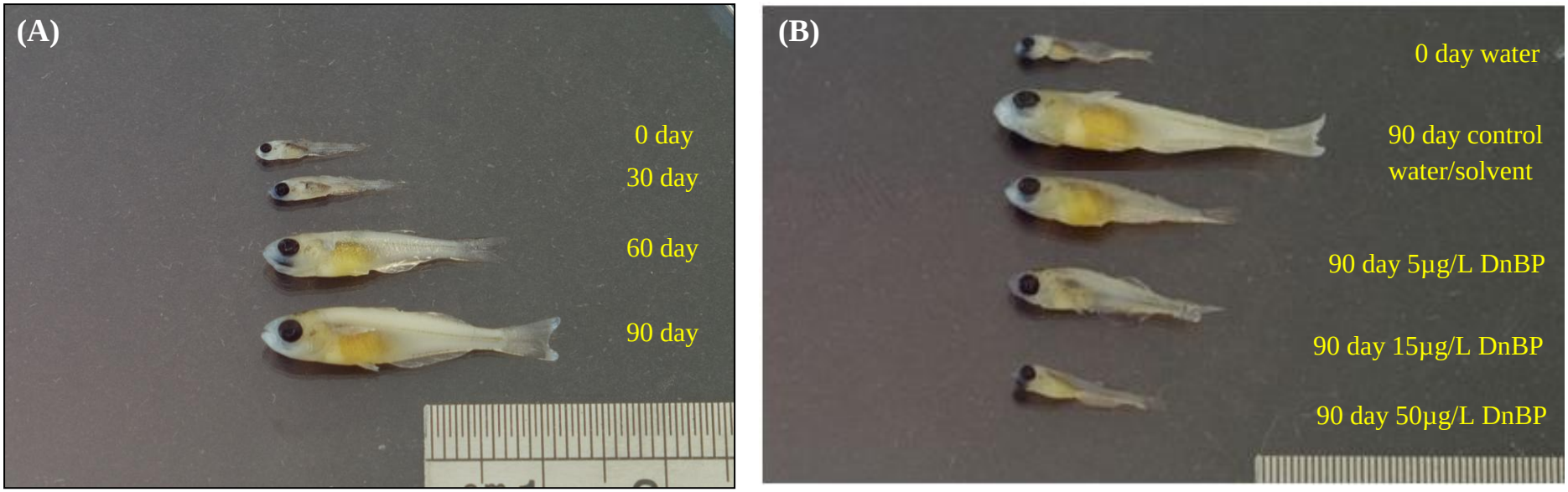
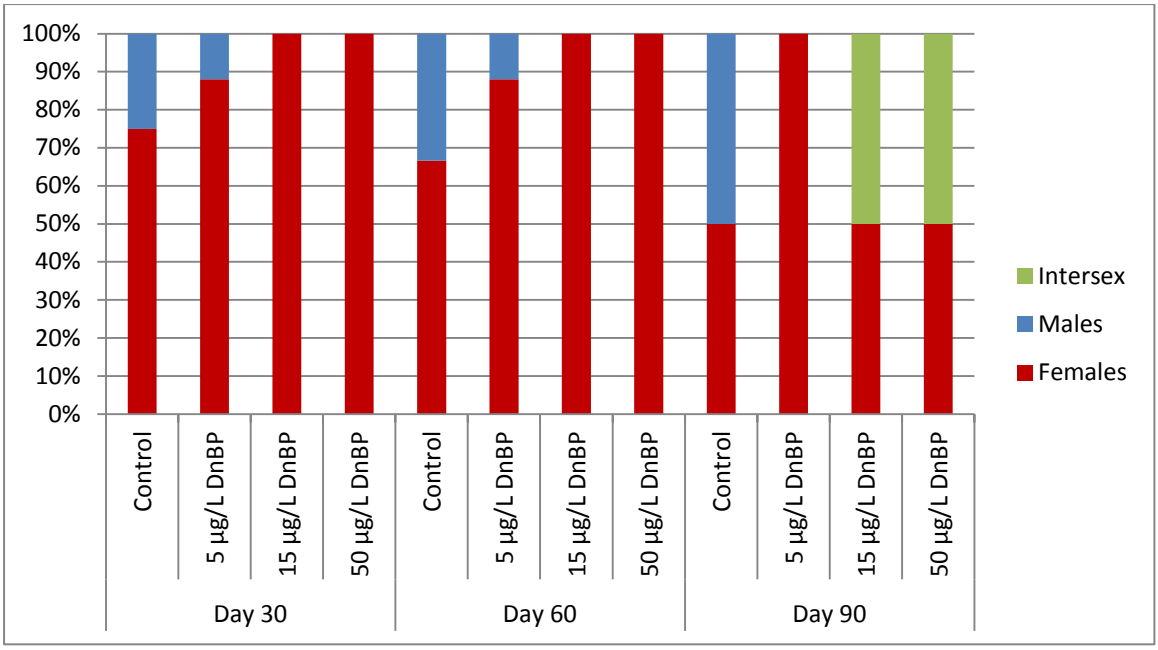

E2 (pg/g fish)
40000
35000
30000
25000
20000
15000
10000
5000
0
Day 0
Day 30
Day 60
Day 90
k
a
a
a
a
a
a
a
ka
kd
kb
kb
e
e
kb
c
e
Water
Solvent
5
15
50
DnBP (µg/L)
11-KT (pg/g fish)
800
700
600
500
400
300
200
100
0
Day 0
Day 30
Day 60
Day 90
k
a
a
a
a
a
a
a
ka
d
kb
c
kb
a
c
kabcd
Water
Solvent
5
15
50
DnBP (µg/L)
E2 / 11-KT
300
250
200
150
100
50
0
Day 0
Day 30
Day 60
Day 90
k
a
b
c
a
b
c
a
bc
d
a
ab
bc
e
ab
bcd
Water
Solvent
5
15
50
DnBP (µg/L)

Figure 5: Whole-body concentrations of (A) 17β-Estradiol (E2) (B) 11-Ketotestosterone (11-KT) and (C) E2/11-KT in juvenile Murray rainbowfish after exposures to 5, 15 and 50 µg/L DnBP for 30, 60 or 90 days each. Bars represent standard error. Bars with different letters are significantly different ($p \leq 0.05$) (n=8).



Supplementary figure 1: Representative growth pattern of juvenile Murray rainbowfish A) in the water control group on days 0, 30, 60 and 90 of the test and B) water control on day 0; water control, solvent control; and 5, 15 and 50 µg/L DnBP treatments on day 90.



Supplementary figure 2: Proportion of sexes in juvenile Murray rainbowfish after exposures to 5, 15 and 50 µg/L di-n-butyl phthalate for up to 90 days.

Table 1. Measured concentrations (mean ± standard error) of di-n-butyl phthalate (DnBP) in fish tanks by gas chromatography-mass spectroscopy (GC-MS) before the start of the fish exposure (n = 2) and 24 h after renewals and spiking of test solutions of DnBP on days 1, 7, 14, 28, 35, 42, 49, 56, 63, 70, 77, 84 and 90 (n = 4) of the test and average water quality parameters (mean ± standard error) measured daily in fish tanks (n = 4).

Treatment	Measured DnBP before the start of the exposure (µg/L)	Measured DnBP 24 h after renewals (µg/L)	pH	DO (%)	Conductivity (µS/cm)	Temperature (°C)
Water	< LOD[a]	< LOQ[b]	7.0 ± 0.5	82.3 ± 3.6	1231 ± 72	23.1 ± 0.1
Solvent	< LOD[a]	< LOQ[b]	7.0 ± 0.5	82.2 ± 4.8	1235 ± 76	
5 µg/L DnBP	5 ± 0.5	6 ± 0.7	6.9 ± 0.5	81.8 ± 4.8	1216 ± 82	
15 µg/L DnBP	15 ± 1	12 ± 2	7.1 ± 0.5	83.2 ± 4.5	1207 ± 86	
50 µg/L DnBP	50 ± 6	36 ± 3	6.8 ± 0.5	80.9 ± 4.9	1241 ± 86	

[a]GC-MS limit of detection (LOD) for DnBP = 0.004 µg/L

[b]GC-MS limit of quantification (LOQ)for DnBP = 0.012 µg/L

CHAPTER 8

SUMMARY AND CONCLUSIONS

Since the reports of endocrine disruption in fish thriving in the water receiving estrogenic effluents from waste-water treatment plants (WWTPs) in the UK (Jobling et al., 1998), many robust laboratory studies have investigated the effects of estrogens in fish (Akerblom et al., 2000; Andersen et al., 2003; Andersson et al., 2007; Belt et al., 2003; Woods, Kumar, 2011). However, the effects of endocrine-disrupting compounds (EDCs) that bind to the androgen receptors (ARs) have not been characterized fully and remain elusive in fish. With the detection of anti-androgenic activity in WWTP effluents (Ma et al., 2013; Urbatzka et al., 2007; Zhao et al., 2011), it has been speculated that sexual disruption in wild fish has a multi-causal etiology involving anti-androgens. In addition, it has been proposed that anti-androgens can cause phenotypic feminisation in fish indicative of an estrogenic exposure and (Sohoni and Sumpter 1998). *In vitro* studies have also established that some natural or anthropogenic chemicals recognised to be estrogenic, can act through an anti-androgenic mechanism (Sohoni, Sumpter, 1998). Some research has been done on fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), three-spined stickleback (*Gasterosteus aculetaus*), mosquitofish (*Gambusia affinis*) and guppy (*Poecilia reticulata*). However, these are introduced species and not native to Australia. There was a need to investigate the effects of anti-androgens on the biomarkers of reproduction in an Australian fresh-water fish species like Murray rainbowfish (*Melanotaenia fluviatilis*).

In the present study, the pharmaceutical anti-androgen, flutamide and an emerging industrial pollutant with anti-androgen properties, di-n-butyl phthalate were chosen to investigate the effects of anti-androgens on some of the biomarkers of reproduction in Murray rainbowfish.

8.1 SUMMARY OF THE RESEARCH

8.1.1 Effects of exposures to the pharmaceutical anti-androgen, flutamide on the biomarkers of reproduction in male, female and juvenile Murray rainbowfish.

Effects of the anti-androgen, flutamide on the biomarkers of reproduction in adult male and female; and juvenile fish were investigated.

8.1.1.1 Testing null hypotheses

Experiments were designed to test the following null hypotheses –

a) The response to short-term exposures to biologically active concentrations of flutamide is equal to the background response in unexposed sexually mature Murray rainbowfish under laboratory exposures.

b) Short-term exposures to biologically active concentrations of flutamide elicit similar responses in sexually mature, adult male and female rainbowfish.

c) Exposures to the commercial anti-androgen, flutamide, alone and in combination, with the reference estrogen, 17β-estradiol (E2) have no effect on the reproductive endocrine function and sexual development of juvenile Murray rainbowfish.

8.1.1.2 Summary of the results

The summary of the major results is given below.

a) Effects of exposures to biologically active concentrations of flutamide for 7 days to biomarkers of reproduction in adult male rainbowfish (Chapter 2)

Adult male rainbowfish were exposed continuously to 125 – 1000 µg/L flutamide in a semi-static set-up involving 100% renewals of the tank water and spiking of fresh test solutions every 24 h. At the end of 7 days, the hepatosomatic index of the fish increased after treatment with flutamide at all concentrations. The transformation of spermatogonia to the successive stages of development was hindered, resulting in increased thickness of the germinal epithelium after treatment with 125 – 1000 µg/L flutamide. In addition, testes of the flutamide-treated fish developed pyknotic and multinucleated cells; and interstitial fibrosis. An induction in the plasma VTG was noted in the 1000 µg/L of flutamide treatment. The brain aromatase activity was reduced after exposures to 125 – 1000 µg/L of flutamide. An up-regulation in the relative hepatic expression of the genes for estrogen receptor α and down-regulation in gene for VTG was noted after treatments with 250 – 1000 µg/L of flutamide treatments. The expression of the gene encoding ARα was down-regulated after exposure to 1000 µg/L and that of ARβ was down-regulated at 500 and 1000 µg/L of flutamide treatments.

b) Effects of exposures to biologically active concentrations of flutamide for 7 days to biomarkers of reproduction in adult female rainbowfish (Chapter 3)

Adult female rainbowfish were exposed to 125 – 1000 µg/L flutamide for 7 days in a semi-static set-up involving 100% renewals of the tank water and spiking of fresh test solutions every 24 h. At the end of the exposure period, the condition factor of fish was reduced in the highest treatment group. The ooplasm was shrunken and the plasma membrane developed undulations. Yolk production was impaired; and multinucleated cysts and interstitial fibrosis were noted in the ovaries of the fish treated with flutamide at all concentrations. The ovaries of the fish treated with higher concentrations of flutamide did not contain mature oocytes. Reductions in the sizes of the early vitellogenic oocytes in treatments of 250 and 500 µg/L of flutamide; and decrease in size of the late vitellogenic oocytes in 500 and 1000 µg/L of flutamide were noted. The brain aromatase activity was reduced in 500 and 1000 µg/L treatments. The plasma E2/11-KT ratio declined at all concentrations tested. Reductions in VTG levels were noted in 125, 500 and 1000 µg/L of flutamide treatments.

c) Effects of exposures to flutamide, on 17β-estradiol induced effects in juvenile rainbowfish over 35-day exposure period (Chapter 4)

Based on the values of anti-androgenic activity detected world-wide, juvenile rainbowfish were exposed to 25 µg/L (low) or 250 µg/L (high) of

flutamide alone and in combination with 25 ng/L of 17β-estradiol (E2) for 35 days in a semi-static set-up involving 100% renewals of the tank water and spiking of fresh test solutions every 24 h. Co-treatment with Flu high and E2 resulted in significant reductions in weights and lengths in males and condition factor in females. Inter-sex was noted in Flu high and E2+Flu high treated fish. The development of spermatocytes in the testes was inhibited by E2 and this effect was accentuated after co-treatment with flutamide. Exposures to E2 resulted in precocious oocyte development in the ovaries which was further up-regulated when fish were co-exposed to E2 and flutamide. The E2 levels decreased significantly in the head of both males and females after co-exposures to flutamide and E2. Flutamide and E2 alone increased the 11-KT levels in both sexes. However, E2+Flu low decreased 11-KT levels in males and increased them in females. Flutamide (low and high) induced VTG protein in the tails of both sexes. In males, VTG was induced in the tail tissue after exposure to flutamide but not E2. No significant increase of flutamide on E2-induced VTG concentration was noted.

8.1.1.3 Alternate hypotheses accepted

Based on the results of the experiments in the present study, the null hypotheses were rejected. The following alternate hypotheses were accepted –

a) The response to short-term exposures to biologically active concentrations of flutamide is not equal to the background response in unexposed sexually mature Murray rainbowfish under laboratory exposures.

b) Short-term exposures to biologically active concentrations of flutamide elicit responses with different MoAs in sexually mature, adult male and female rainbowfish.

c) Exposures to the commercial anti-androgen, flutamide, alone and in combination, with the reference estrogen, E2 affect the reproductive endocrine function and sexual development of juvenile Murray rainbowfish.

8.1.2 Effects of exposures to di-n-butyl phthalate on the biomarkers of reproduction in male, female and juvenile Murray rainbowfish.

Effects of the industrial plasticiser, DnBP on the biomarkers of reproduction, in particular anti-androgenic effects, in adult male and female; and juvenile fish were investigated.

8.1.2.1 Testing null hypotheses

Experiments were designed to test the following null hypotheses –

a) The response to short-term exposures to sub-acute concentrations of DnBP is equal to the background response in unexposed sexually mature Murray rainbowfish under laboratory exposures.

b) Short-term exposures to biologically active concentrations of DnBP elicit similar responses in sexually mature, adult male and female rainbowfish.

c) Long-term chronic exposures to the environmentally relevant concentrations of DnBP have no effect on growth and gonadal development in juvenile Murray rainbowfish.

8.1.2.2 Summary of the results

Summary of the results is given below.

a) Effects of exposures to sub-acute concentrations of DnBP for 7 days in adult male rainbowfish (Chapter 5)

Adult male rainbowfish were exposed continuously to 125 – 1000 µg/L DnBP for 7 days in a semi-static set-up involving 100% renewals of the tank water and spiking of fresh test solutions every 24 h. At the end of the exposure period, the sizes of spermatogonia, spermatocytes and spermatids were reduced in 125 – 1000 µg/L of DnBP. In addition, the testes showed the presence of vacuolated cells, apoptotic cell bodies, interstitial fibrosis and asynchronous development in 1000 µg/L of DnBP treated-fish. There was an increase in the plasma VTG in 500 and 1000 µg/L and brain aromatase activity after exposure of fish to 1000 µg/L DnBP. The hepatic expression of ER mRNA was induced. The VTG transcript was significantly induced in the lowest treatment; and that of choriogenin-L (ChG-L) mRNA and AR mRNA were induced in the highest treatment.

b) Effects of exposures to sub-acute concentrations of DnBP for 7 days in adult female rainbowfish (Chapter 6)

Adult female rainbowfish were exposed continuously to 125 – 1000 µg/L DnBP for 7 days in a semi-static set-up involving 100% renewals of the tank water and spiking of fresh test solutions every 24 h. At the end of the exposure period, increases in the sizes of previtellogenic oocytes were noted in 250 – 1000 µg/L of DnBP treatments and reduction in the sizes of early vitellogenic oocytes was noted after treatment with 1000 µg/L DnBP. Impaired yolk formation, granulomatous inflammation, cysts, interstitial fibrosis, shrunken ooplasm and granulosa cell hyperplasia were noted in the ovaries of the DnBP-treated fish. This was accompanied by reductions in the thickness of chorion layer. Plasma VTG levels were reduced in 500 and 1000 µg/L of DnBP-treated fish.

c) Effects of exposures to environmentally relevant concentrations of DnBP for 90 days in juvenile Murray rainbowfish (Chapter 7)

Juvenile Murray rainbowfish were exposed continuously to 5, 15 and 50 µg/L DnBP for 30, 60 and 90 days in a semi-static set-up involving 100% renewals of the tank water and spiking of fresh test solutions every 24 h. Exposures to 15 and 50 µg/L of DnBP resulted in reductions in the weights of the fish at all sampling times. Lengths were reduced after 60 days in all DnBP treatments. The lowest observed effective concentration (LOEC) to induce significant changes in CF was 5 µg/L after 90 days. Complete feminisation of the gonads was noted in fish exposed to 5 µg/L DnBP for 90 days and in 15 and 50 µg/L of DnBP treatments at all time intervals investigated with intersex condition in 50% of the fish on day 90. Ovaries in all DnBP-treated fish were regressed and immature containing only perinucleolar

oocytes. Absence of vitellogenic oocytes confirmed that the ovaries were immature. On day 90, a significant increase in E2 and decrease in 11-KT levels were noted in 5 µg/L treatment. Significant inductions in the E2/11-KT levels were measured in fish exposed to 5 µg/L of DnBP for 90 days and in those exposed to 50 µg/L of DnBP for 30 days.

8.1.2.3 Alternate hypotheses accepted

Based on the results of the experiments in the present study, the null hypotheses were rejected. The following alternate hypotheses were accepted –

a) The response to short-term exposures to sub-acute concentrations of DnBP is not equal to the background response in unexposed sexually mature Murray rainbowfish under laboratory exposures.

b) Short-term exposures to biologically active concentrations of DnBP elicit responses with different MoA in sexually mature, adult male and female rainbowfish.

c) Long-term chronic exposures to the environmentally relevant concentrations of DnBP, as low as 5 µg/L, affect the growth and gonadal development in juvenile Murray rainbowfish.

8.2 CONCLUSIONS

Based on the analyses and interpretation of the data obtained from the experiments in the present study, the following conclusions are made –

8.2.1 Short-term exposures to biologically active concentrations of flutamide cause anti-androgenic effects in adult male and defeminising effects in adult female Murray rainbowfish.

Short-term exposures to biologically active concentrations of flutamide down-regulated the expression levels of the genes for ARα and ARβ suggesting an anti-androgenic mode of action of flutamide in male fish. In addition, induction of VTG levels and increase in the expression levels of ERs suggest feminising effects. On the other hand, flutamide induced defeminising effects like reduction in the brain aromatase activity, lower levels of plasma E2, reduction in VTG production and failure of the oocytes to mature in adult female fish.

Based on the discussion from the present study, the following hypothetical models of effects of flutamide in adult male and female fish are proposed -

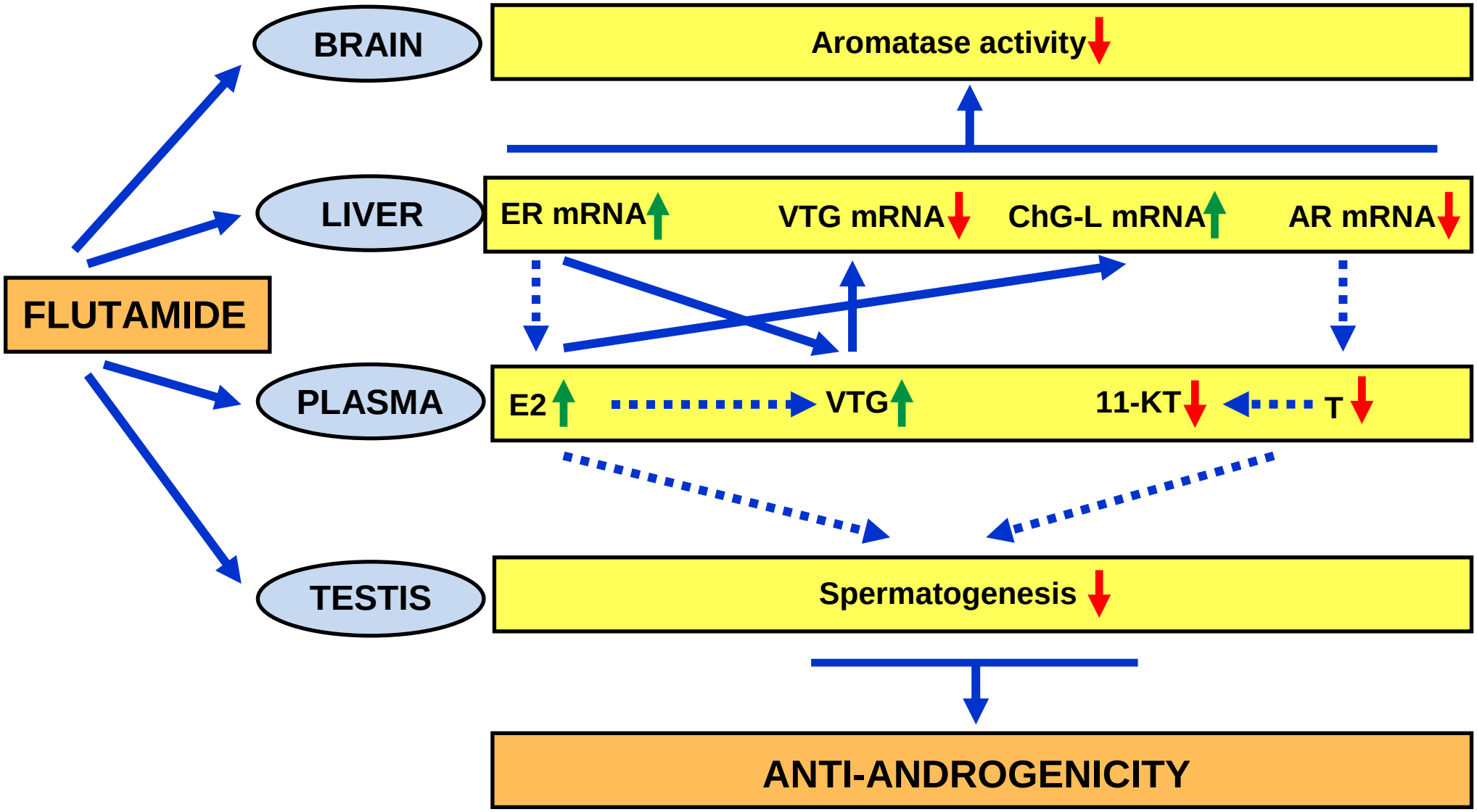


Figure 1: Hypothetical model of biological effects of flutamide in adult male Murray rainbowfish. Green arrows represent up-regulation and red arrows represent down-regulation based on the data from the present study. Broken blue arrows represent plausible effect based on previous studies and solid blue arrows are data from the present study. E2: 17β-estradiol; 11-KT: 11-keto testosterone; VTG: Vitellogenin; ER: Estrogen receptor; AR: Androgen receptor; ChG-L: Choriogenin-L; T: Testosterone

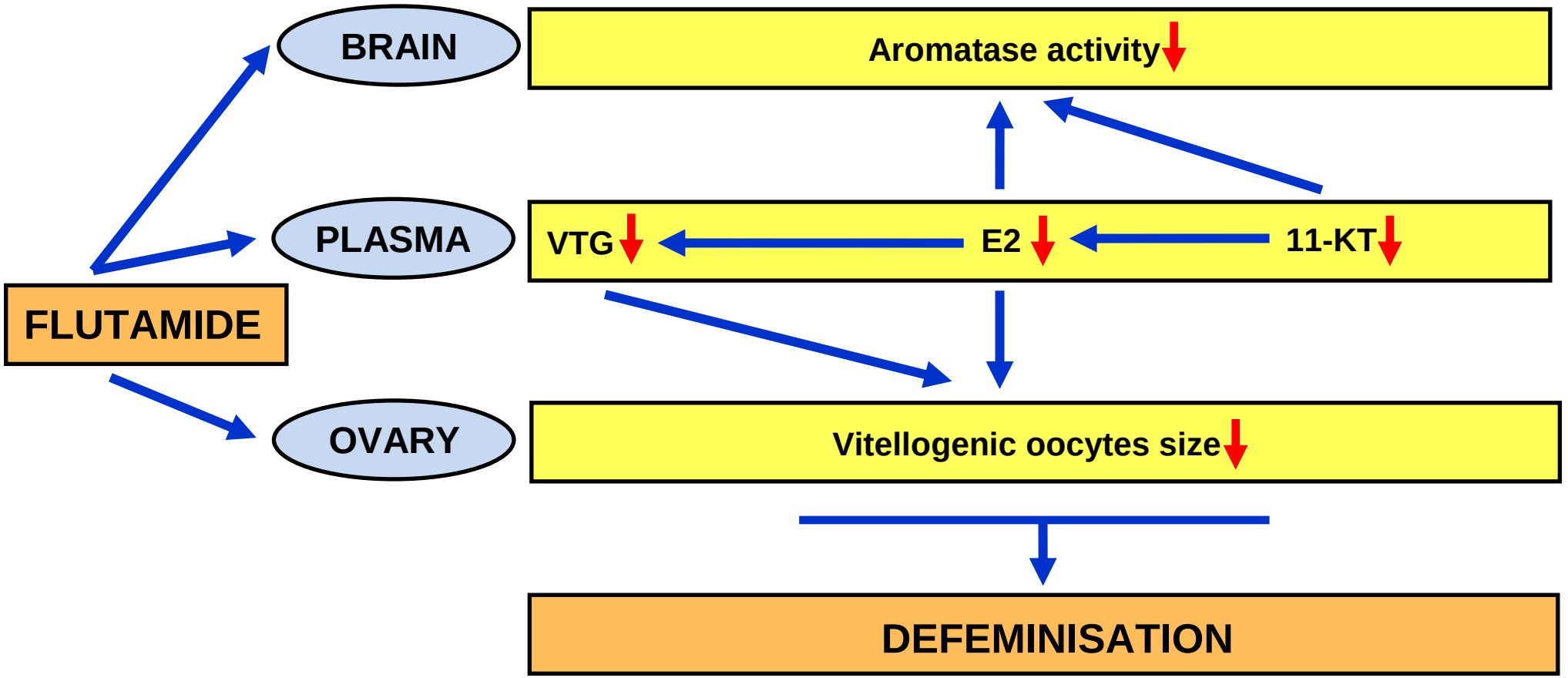


Figure 2: Hypothetical model of biological effects of flutamide in adult female Murray rainbowfish. Red arrows represent down-regulation based on the data from the present study. Soli blue arrows are the data from the present study. E2: 17β-estradiol; 11-KT: 11-keto testosterone; VTG: Vitellogenin

8.2.2 Exposure to estrogens can cause phenotypic feminisation in sexually undifferentiated, juvenile Murray rainbowfish and these effects are elevated by the co-treatment with anti-androgens.

The present study confirms previous *in vitro* findings that anti-androgens can cause phenotypic feminisation indicative of an estrogenic exposure. The present study confirms previous *in vivo* findings that chemicals identified as anti-androgens like flutamide can interfere with the reproductive fitness in fish. We found that flutamide asserts similar effects as E2 on the biochemical, histological and hormonal profile of juvenile fish. In addition, flutamide did not significantly increase the E2-induced effects on body-growth; and the concentrations of VTG and sex-steroid hormones. However, qualitative investigation showed that flutamide amplified the E2-induced effects on gonadal cell development in juvenile fish. E2+flu high inhibited the sperm development in the testes and resulted in precocious oocyte development in the ovaries. Based on the previous studies and the data from the present study, we conclude that anti-androgens do not add to the effects of estrogens due to different modes of action. However, they induce similar effects which can cause additive inhibition/stimulation of the gonad development. The incidence of intersex highlights the potential adverse effects of environmentally relevant concentrations of anti-androgens in combination with estrogens on sexual differentiation of juvenile fish.

Based on the discussion from the present study, the following models of effects of flutamide alone and in combination with E2 in juvenile male and female fish are proposed -

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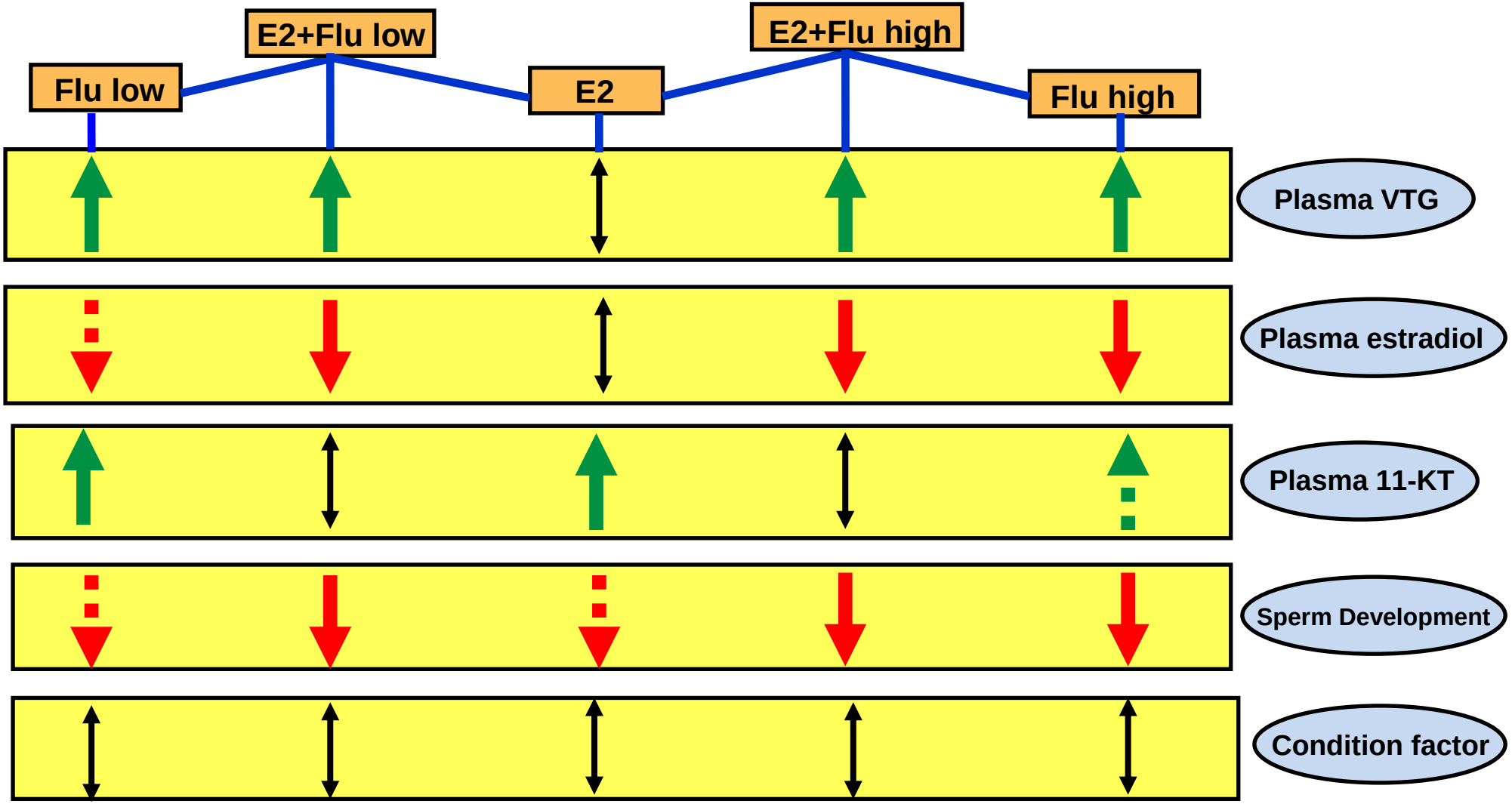

Figure 3: Hypothetical model of biological effects of flutamide in juvenile male Murray rainbowfish. Green arrows represent up-regulation and red arrows represent down-regulation based on the data from the present study. Higher the intensity /thickness of the arrow, higher is the effect. Double-headed arrows represent no effect. Flu low: 25µg/L flutamide; Flu high: 250 µg/L flutamide; E2: 25 ng/L 17β-estradiol; VTG: Vitellogenin; 11-KT: 11-Keto testosterone; CF: Condition factor.

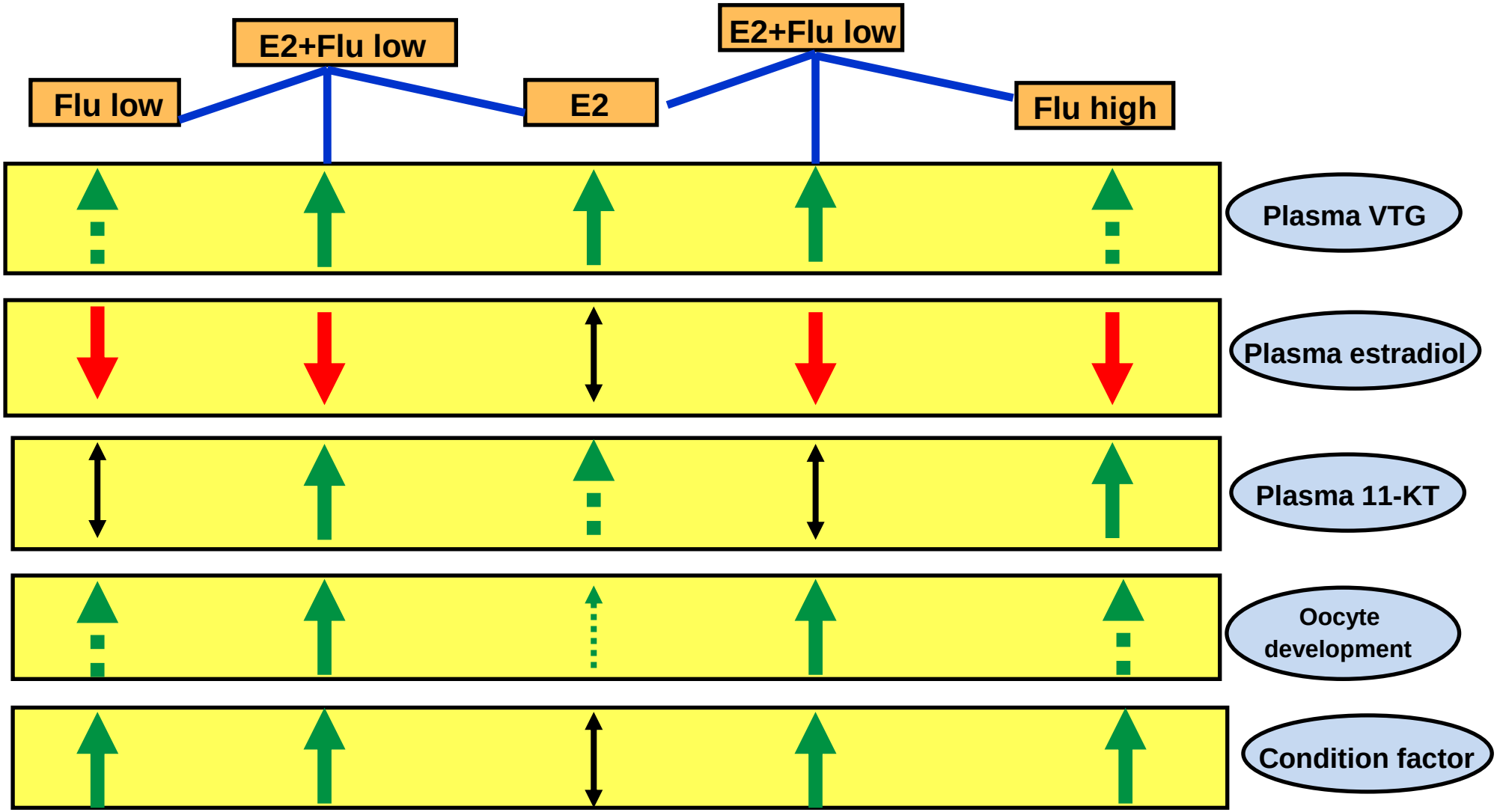

Figure 4: Hypothetical model of biological effects of flutamide in juvenile female Murray rainbowfish. Green arrows represent up-regulation and red arrows represent down-regulation based on the data from the present study. Higher the intensity /thickness of the arrow, higher is the effect. Double-headed arrows represent no effect. Flu low: 25µg/L flutamide; Flu high: 250 µg/L flutamide; E2: 25 ng/L 17β-estradiol; VTG: Vitellogenin; 11-KT: 11-Keto testosterone; CF: Condition factor.

8.2.3 Short-term exposures to sub-acute concentrations of DnBP cause anti-estrogenic effects in adult female and estrogenic effects in adult male Murray rainbowfish.

Exposures to sub-acute concentrations of DnBP for 7 days can result defeminisation of adult female fish as evident from reductions in the sizes of vitellogenic oocytes and circulating levels of VTG. However in adult male fish, similar exposures induce VTG protein and the expression of the genes encoding ERs suggested estrogenic effects.

Based on the discussion from the present study, the following models of effects of DnBP in adult fish are proposed -

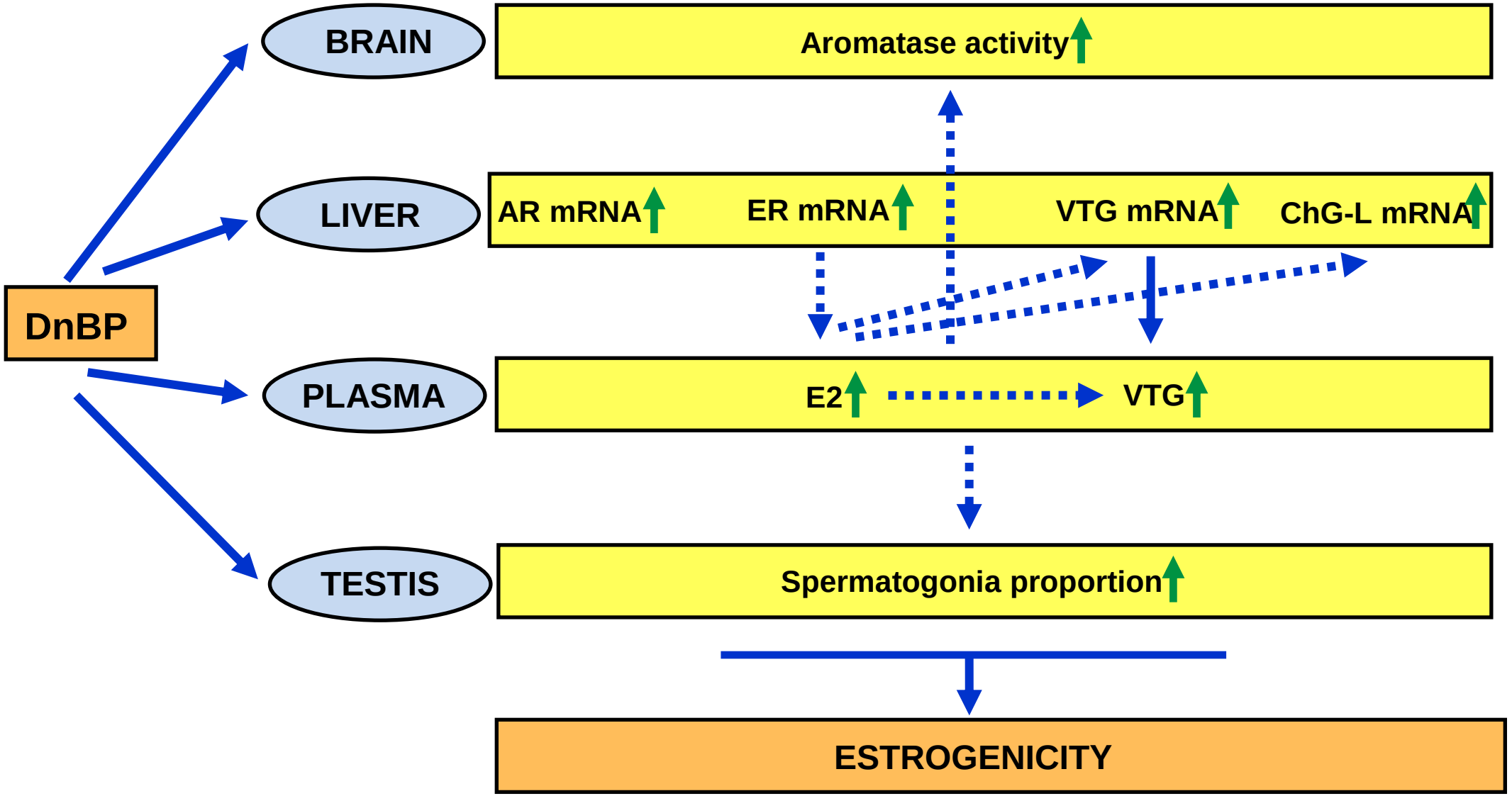

Figure 5: Hypothetical model of biological effects of DnBP in adult male Murray rainbowfish. Green arrows represent up-regulation based on the data from the present study. Broken blue arrows represent plausible effect based on previous studies. DnBP: Di-n-butyl phthalate; AR: Androgen receptor; ER: Estrogen receptor; VTG: Vitellogenin; ChG-L: Choriogenin-L; E2: 17β-Estradiol; 11-KT: 11-Keto testosterone; ChG-L: Choriogenin-L

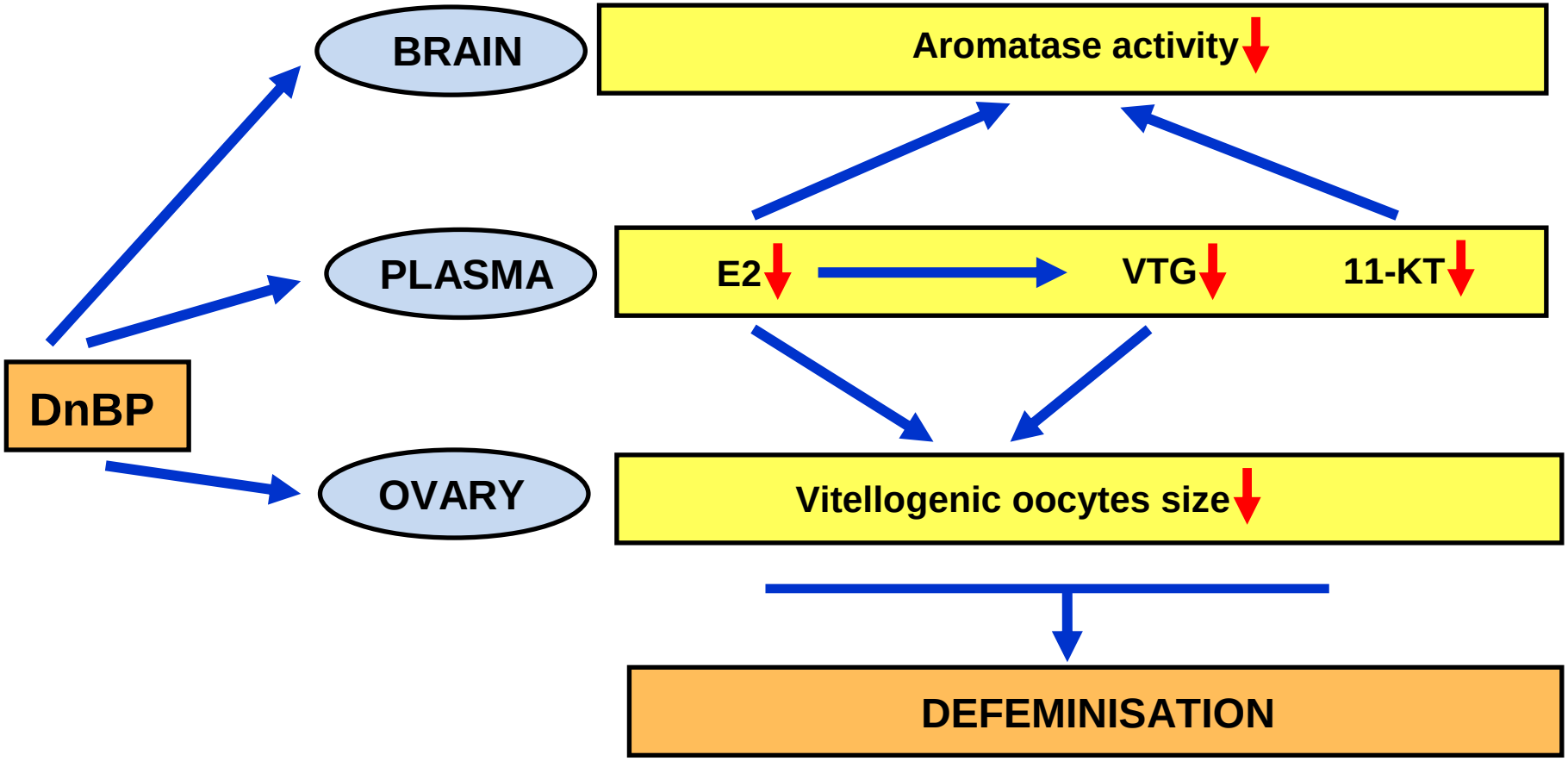

Figure 6: Hypothetical model of biological effects of DnBP in adult female Murray rainbowfish. Red arrows represent down-regulation based on the data from the present study. DnBP: Di-n-butyl phthalate; E2: 17β-Estradiol; VTG: Vitellogenin; 11-KT: 11-Ketosterone

8.2.4 Short-term exposure to high concentrations and long-term exposure to low concentrations of DnBP have similar hormonal effects on the reproductive endocrine function in juvenile Murray rainbowfish.

Estrogenicity, as divulged from the E2/11-KT values, was induced in juvenile rainbowfish after exposures to 5 µg/L of DnBP for 90 d and 50 µg/L of DnBP for 30 days. Significant adverse effects on the hormonal profile and histological development of the gonads were noted after short-term exposures to high concentrations and long-term exposures to low concentrations of DnBP. Based on the discussion in the present study, the following hypothetical model of effects of DnBP in juvenile fish has been proposed -

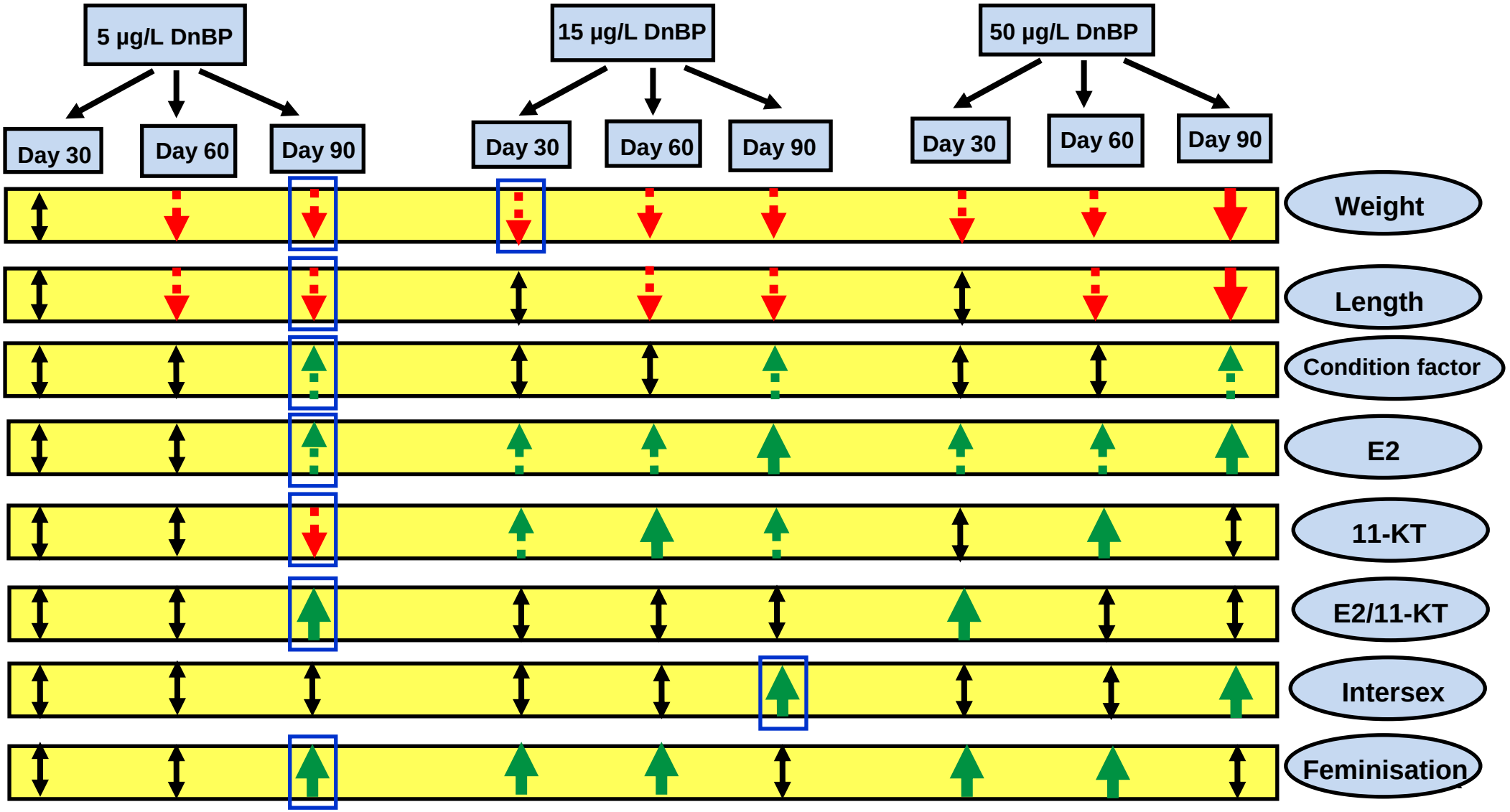

Figure 7: Hypothetical model of biological effects of DnBP in juvenile Murray rainbowfish. Green arrows represent up-regulation and red arrows represent down-regulation based on the data from the present study. Double-headed arrows represent no effect. The effects representing 90 day LOEC values have been highlighted with a blue box. The solid and dashed arrows are significantly different. DnBP: Di-n-butyl phthalate; E2: 17β-Estradiol; VTG: Vitellogenin; 11-KT: 11-Ketosterone; CF: Condition factor

8.2.5 Levels of *in vitro* anti-androgenic activity detected in fresh-water worldwide could impair reproductive fitness of fish.

Flutamide is a classic anti-androgen and is used to treat prostate cancer in men and polycystic ovarian syndrome in women. However, flutamide has not been detected in the Australian riverine environment. It is extensively used in toxicity testing in mammals since it is a "pure" anti-androgen. For the same reason, the anti-androgenic activity determined by *in vitro* assays, is expressed in flutamide equivalency (FEQ). The total anti-androgenic activity (expressed in flutamide equivalents) in freshwater worldwide has been reported to range between 100 µg FEQ/L to 1000 µg FEQ/L. The biological effects we observed in the present study in adult male and female fish after exposures to 250 – 1000 µg/L flutamide for 7 days and in juvenile fish after exposures to 25 or 250 µg/L of flutamide could be of concern for the reproductive health of the fish thriving in the receiving environment containing high anti-androgenic activity.

8.2.6 Levels of DnBP found in the freshwater in Australia, do not pose a threat to the reproductive fitness of Murray rainbowfish. However, there is a strong need to revise the water quality guidelines for DnBP in fresh-water in Australia.

The results from the present study raise concerns for phthalates as emerging endocrine-disrupting chemicals that can pose threat to the reproductive health of the fish thriving in heavily polluted water-bodies. The concentrations of DnBP (250 – 1000 µg/L) at which the effects were found in sexually mature, adult fish were higher than even the peak concentrations of phthalates measured in the Australian aquatic

environment. The concentration of phthalates in the fresh-water environment of Australia and worldwide is usually below 100 µg/L. We recommend that the concentrations of DnBP tested in the present study using sexually mature, adult fish are likely levels of phthalates in the aquatic environment (generally within a factor of 10) and are close to or even overlap the acute levels at which other toxic effects may by exerted over a longer exposure period, thus corroborating a risk to fish thriving in heavily polluted water bodies. Currently, Australia recommends maximum of 9.9 µg/L of DnBP for freshwater ecosystem (http://www.environment.gov.au/resource/australian-and-new-zealand-guidelines-fresh-and-marine-water-quality-volume-1-guidelines). The 90 day-LOEC values of DnBP for survival (> 50 µg/L), length (> 5 µg/L), weight (> 5 µg/L) and body condition factor (> 5 µg/L) proposed in the present study using juvenile fish are below the water quality guidelines and suggest the possible population-relevant adverse effects at concentrations below the water quality guidelines. The general health, gonadal development and sex-steroid hormone profile of the juvenile fish were adversely affected after treatments with low concentrations of DnBP (5 µg/L) for longer periods (90 days). Although the levels of DnBP detected in Australian environment (47 ng/L) (Tan et al., 2007) are safe (based on the LC-50 values reported in previous studies and included as Table 9 in the Introduction of this thesis), the water-quality guidelines are not protective for Murray rainbowfish. The Australia and New Zealand guidelines (ANZECC/ARMCANZ, 2000) stipulated that for guidance values derivation, only toxicity data that measured survival, growth and reproduction were acceptable. Biomarker or most behavioural data were not used due to their lack of proven ecological relevance. However, Canadian guidelines (CCME, 2007) admit traditional endpoints like growth, reproduction and survival as well as non-traditional endpoints like behaviour, predator avoidance, swimming ability,

swimming speed, etc. and physiological/biochemical changes like endocrine-disrupting ability can be used if they impact a species' ecological competitiveness and lead to an ecologically relevant negative impact (they affect traditional endpoints).

8.2.7 The mammalian anti-androgen, DnBP and the commercial anti-androgen, flutamide assert similar effects but with different mechanisms in Murray rainbowfish

In the present study, DnBP exposures caused estrogenic effects in adult male fish (as evident from induction of the genes for ERs and ARs, circulating levels of VTG protein and increase in aromatase activity and interstitial fibrosis in the testes). After exposures to flutamide, although the testicular histopathological effects were similar, decrease in aromatase activity and reduction in the expression levels of the genes encoding ARs was noted. In females, DnBP induced defeminisation of the fish. The sizes of the pre-vitellogenic oocytes were larger and those of the vitellogenic oocytes were smaller. This was associated with reductions in the circulating levels of VTG. Similarly, in females after flutamide exposure, defeminisation was noted. There was reduction in the circulating levels of VTG and E2. This was accompanied by reductions in the sizes of the oocytes and absence of mature oocytes in flutamide-treated fish.

8.3 RECOMMENDATIONS FOR FUTURE WORK

The present project was designed to investigate the reproductive endocrine effects of the commercial anti-androgen, flutamide and the industrial pollutant, DnBP in adult (male and female) and sexually undifferentiated juvenile Murray rainbowfish

at multiple levels of organisation. While it was concluded that both chemicals have gender and age-specific modes of action and the effects, some areas have been identified that require further knowledge. Based on the results collated from the present study, the following investigations from an Australian and international perspective are recommended for the future studies.

8.3.1 Investigation of the reproductive endocrine effects of DnBP at environmentally relevant concentrations in sexually mature, adult Murray rainbowfish is needed.

In the present study, we exposed sexually mature male and female fish to sub-acute concentrations of DnBP to identify the effects and elucidate the *in vivo* mechanism of action of phthalates in fresh-water. Further studies involving exposure of sexually mature, adult fish to environmentally relevant concentrations of DnBP over a longer period of time can help in better understanding of the effects of phthalates in the fresh-water environment. In addition, research on the interactions of DnBP with other contaminants is needed because phthalates are likely to be found in complex mixtures in the receiving environment.

8.3.2 Detection, identification and quantification of the individual anti-androgens in fresh-water environment in Australia and world-wide are needed.

Although *in vitro* assays have detected 100 – 1000 FEQ/L anti-androgenic activity in the aquatic environments (effluents and receiving environments) worldwide, there is a need to identify, detect and quantify the individual anti-

androgens. Further laboratory testing of these anti-androgens under controlled conditions using robust experiments will better understand the extent and mechanisms of endocrine disruption in wild fish caused by anti-androgens.

8.3.3 Identification of the genes and testing of the molecular tools regulating gonadal differentiation in Murray rainbowfish are needed.

It is important to identify the male- and female-related genes involved in histogenesis (like *Dmrt1*, *Dmy*, *Foxl2* etc) during sex differentiation in Murray rainbowfish to better understand the mechanisms regulating gonadal sex differentiation in juvenile Murray rainbowfish. This will help to validate the effects of endocrine disrupting chemicals at the genetic level in fish. In addition, testing of these molecular tools in other rainbowfish species will further enhance the use of Murray rainbowfish use as a fresh-water model test species across Australia.

8.3.4 Investigation into the recovery of the juvenile fish after exposures to environmentally relevant concentrations of DnBP is needed.

Exposure to 50 µg/L of DnBP for 30 days induced the E2/11-KT ratio in the body of the juvenile fish. After 90 days of exposure, the ratio was comparable to the corresponding fish in the 90 days controls. In addition, complete feminisation of the gonads was seen after exposures to 15 µg/L and 50 µg//L of DnBP for 30, 60 and 90 days. However, on day 90, intersex condition was seen in the fish exposed to 50 µg/l of DnBP. The gonads contained both spermatocytes and perinucleolar oocytes, suggesting different spectra of effects. It would be useful to investigate the

reversibility of the effects of phthalates after chronic exposures in juvenile fish. In addition, analyses of the expression levels of the genes involved in genetic sex-determination like *Dmrt1*, *Dmy* (testicular differentiation) and *cyp19a*, *Foxl2* (ovarian differentiation) can help better understand if the incidences of sex reversal or intersex gonads in sexually undifferentiated fish are permanent.

8.3.5 Fish caging studies in the receiving environment are needed.

The EDCs are not fully removed from water by the WWTPs. These chemicals are discharged into fresh-water in very minute amounts (parts per billion or parts per trillion). The receiving environment contains a cocktail of chemicals with different MoAs (androgenic, anti-androgenic, estrogenic, anti-estrogenic). It is important to investigate the endocrine disruption, if any, in the wild fish thriving in this aquatic environment in Australia. Similar studies have been reported in Europe and the US. These studies can be conducted by using mobile fish caging units in the receiving environment. In addition, wild Murray rainbowfish thriving in the Murray Darling river basin receiving treated effluents from the WWTPs should be assessed for reproductive endocrine disruption, if any.

8.4 REFERENCES

Akerblom, N., Olsson, K., Berg, A. H., Andersson, P. L., Tysklind, M., Forlin, L. and Norrgren, L. 2000. Impact of polychlorinated naphthalenes (PCNs) in juvenile Baltic salmon, *Salmo salar*: Evaluation of estrogenic effects, development, and CYP1a induction. Archives of Environmental Contamination and Toxicology 38, 225-233.

Andersen, L., Holbech, H., Gessbo, A., Norrgren, L and Petersen, G. I. 2003. Effects of exposure to 17 alpha-ethinylestradiol during early development on sexual

differentiation and induction of vitellogenin in zebrafish (*Danio rerio*). Comparative Biochemistry and Physiology C-Toxicology and Pharmacology 134, 365-374.

Andersson, C., Katsiadaki, I., Lundstedt-Enkel, K. and Orberg, J. 2007. Effects of 17 alpha-ethynylestradiol on EROD activity, spiggin and vitellogenin in three-spined stickleback (*Gasterosteus aculeatus*). Aquatic Toxicology 83, 33-42.

Belt, K. V. D., Verheyen, R. and Witters, H. 2003. Effects of 17alpha-ethynylestradiol in a partial life-cycle test with zebrafish (*Danio rerio*): Effects on growth, gonads and female reproductive success. Science of the Total Environment 309, 127-137.

Jobling, S., Nolan, M., Tyler, C. R., Brighty, G and Sumpter, J. P. 1998. Widespread sexual disruption in wild fish. Environmental Science and Technology 32, 2498-2506.

Ma, D., Chen, L. and Lui, R. 2013. Decrease of antiandrogenic activity in gray water and domestic wastewater treated by the mbr process. Environmental Science-Processes and Impacts 15, 668-676.

Sohoni, P. and Sumpter, J. P. 1998. Several environmental oestrogens are also anti-androgens. Journal of Endocrinology 158, 327-339.

Tan, B. L. L., Hawker, D. W., Muller, J. F., Leusch, F. D. L., Tremblay, L. A. and Chapman, H. F. 2007. Comprehensive study of endocrine disrupting compounds using grab and passive sampling at selected wastewater treatment plants in south east queensland, australia. Environment International 33, 654-669.

Urbatzka, R., Bottero, S., Mandich, A., Lutz, I. and Kloas, W. 2007. Endocrine disrupters with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of *Xenopus laevis*: I. Effects on sex steroid levels and biomarker expression. Comparative Biochemistry and Physiology C-Toxicology and Pharmacology 144, 310-318.

Woods, M. and Kumar, A. 2011. Vitellogenin induction by 17beta-ethynylestradiol in male murray rainbowfish (*Melanotaenia fluviatilis*). Environmental Toxicology and Chemistry 30, 2620-2627.

Zhao, J. L., Ying, G. G., Yang, B., Liu, S., Zhou, L. J., Chen, Z. F. and Lai, H. J. 2011. Screening of multiple hormonal activities in surface water and sediment from the Pearl river system, South China, using effect-directed *in vitro* bioassays. Environmental Toxicology and Chemistry 30, 2208-2215.