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*)

Harpreet Bhatia

B.Sc. (Life Science); **M.Sc.** (Zoology); **B.Ed.** (Teaching of Science);
Grad. Cert. (Medical Biotechnology)

Submitted for the degree of Doctor of Philosophy

October 2014

School of Agriculture, Food and Wine
The University of Adelaide

To

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

and

My husband
For making me wonder who I am

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

This thesis not only represents my work in the laboratory and on the keyboard, it is a milestone for and also belongs to the Aquatic Toxicology research team at CSIRO. Since my first day at CSIRO on May 10, 2010, I have been given unique opportunities, which I have grabbed with both hands. This thesis is the result of my amazing experiences from dozens of remarkable people who I wish to acknowledge.

First and foremost I owe my deepest gratitude to my supervisor, Dr. Anu Kumar. Anu has supported me not only by providing research infrastructure and funding for four years, but also academically and emotionally through the rough road in shaping my project and finishing this thesis. During the most difficult times, she gave me moral support and the freedom to move on. I cherish the rapport and friendship we share. Her resilient belief in me and a pat on the back have been my fuel to move forward. I remember she used to say, “You are nearly there Harpreet and I know you can do it!"

My heartfelt appreciation goes to my thesis advisory panel members – Prof. Mike J. McLaughlin and Dr. John Chapman – for their immense support, prompt feedbacks, expert guidance and cheerful greetings. With your contagious enthusiasm, you have been an inspiration in many ways.

Dozens of people have helped, taught me immensely and shared their skills and suggestions during these years. Thanks to Mr. Hai Doan, Ms. Debra Gonzago and Ms. Bhanu Nidumolu for their help and advices in the laboratory; Ms. Adrienne Gregg for her assistance in the Molecular Biology techniques; Dr. Jun Du for her input in the Analytical Chemistry; Dr. Jason Kirby and Ms. Gill Cozens for their advice in the radioactivity assay; Ms. Rupal Pradhan and Mr. Andrew Beck for their help in histological techniques; Dr. Peter Bain for his insightful discussions and Mr. Chad Badman for his prompt help with printing the thesis. I appreciate the feedback offered by Dr. Graeme Batley on my manuscripts. Particular thanks to Prof. Taisen Iguchi and Dr. Yukiko Ogino for their collaborations, contributions to Molecular Biology, expert interpretation of data and comments on my manuscripts. Special thanks to Dr. Rai Kookana and Dr. Paul Harvey for their inspiring and magnetic presence.

I am thankful to the University of Adelaide for the award of Adelaide Fee Scholarship. I highly appreciate the support from Prof. Richard Russell, Dean of Graduate Studies and Dr. Cameron Grant, Post-graduate coordinator during these years. I acknowledge

the assistance from CSIRO in terms of providing infrastructure and funding for this project. I thank the animal ethics committees from the University of Adelaide and CSIRO facilities for approving the usage of fish in this project and the Aquarium Industries, Victoria for their supply of fish. I also acknowledge the University of South Australia for their histology facilities and the Adelaide Microscopy, North Terrace for their image analysis software.

I feel short of words in acknowledging my best friend and husband, Inderpreet Singh Gill, having achieved much more in life than this thesis. I am thankful to you more than you can imagine. I am sorry for all the late evenings, busy weekends and for not being always there for you in these four years. Thank you for understanding my professional commitments, supporting me immensely, pulling me out of disasters and helping me become a better human being with your sheer optimism. I would not have achieved this without you being by my side.

I dedicate this thesis to my father, S. Satinder Singh and to my mother, Sdn. Harvinder Kaur. Thank you, Papa and Mumma for instilling in me the love for books. Mumma, I know you are happy and healthy in heaven. I talk to you everyday and I will meet you some day. Continue to bless me at every step so I am able to fulfil all your desires. I love you, I miss you and it is an unbearable pain to not be able to see or listen to you. I hope I made you proud today.

I cherish the lovely company of my dearest brother, Aalam Inder Singh. Thank you, Aalam for loving and caring for me always. Thanks to my sister, Harneet; my parents-in law; and my extended family for being there for me and for my family in the most sensitive phases of our lives. Your care let me focus on my goals when I was the weakest. This day, I fondly feel the love of my grandma, Sdn. Kuldeep Kaur and my late grandparents, S. Gian Singh, Sdn. Harbans Kaur and S. Inder Singh.

I anticipate the sacrifice of the hundreds of fish involved in this project will aid in laying the water quality guidelines in Australia and form the bases of the future ecotoxicological research in environmental contaminants. Last but not the least; I bow my head to Waheguru for giving me physical and mental strength to drive through some of the most difficult phases of life. He has written beautiful stories for everyone and I am thankful for mine.

HARPREET BHATIA

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.

I give consent to this copy of thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

The author acknowledges that copyright of published and soon to be published works contained in this thesis (as listed below) resides with the copyright holder(s) of those works.

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.

2. Harpreet Bhatia, Anupama Kumar, John Chapman and Mike J. McLaughlin (2014) Effects of short-term exposure to the model anti-androgen, flutamide on reproductive function based endpoints in female Murray rainbowfish (*Melanotaenia fluviatilis*). Ecotoxicology and Environmental Safety 109: 143 – 151.
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**

LIST OF TABLES

CHAPTER 1

REVIEW OF LITERATURE

Chapter 2

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

CHAPTER 5

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

CHAPTER 6

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

LIST OF FIGURES

Chapter 1

Review of Literature

Chapter 2

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

CHAPTER 3

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

CHAPTER 4

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

CHAPTER 5

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

CHAPTER 6

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

CHAPTER 7

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

CHAPTER 8

Summary and conclusions

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.
2. **Harpreet Bhatia**, Anupama Kumar, John Chapman and Mike J. McLaughlin (2014) Effects of short-term exposure to the model anti-androgen, flutamide on reproductive function based endpoints in female Murray rainbowfish (*Melanotaenia fluviatilis*). Ecotoxicology and Environmental Safety 109: 143 – 151.
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).

Abstracts

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)

Ovarian histopathology as a tool to evaluate endocrine disruption by Di-n-butyl phthalate in Murray rainbowfish (*Melanotaenia fluviatilis*). Society of environmental toxicology and chemistry conference. Brisbane (Australia) July 4-6, 2012. p.163

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.

   POSTER PRESENTATION

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.

   BEST ORAL PRESENTATION

3. **Harpreet Bhatia**, Anupama Kumar, Yukiko Ogino, Adrienne Gregg, John Chapman, Mike J. McLaughlin, and Taisen Iguchi. Di-n-butyl phthalate causes estrogenic effects in adult male Murray rainbowfish (*Melanotaenia fluviatilis*).

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

BEST ORAL PRESENTATION

4. **Harpreet Bhatia**, Anupama Kumar, Yukiko Ogino, Jun Du, Adrienne Gregg, John Chapman, Mike J. McLaughlin and Taisen Iguchi. Adult male Murray rainbowfish (*Melanotaenia fluviatilis*) as a test model to assess anti-androgenic effects of flutamide in Australian riverine environment.

   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.