

**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

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	vii
Declaration	ix
List of tables	xi
List of figures	xv
List of abbreviations	xxv
List of publications and presentations	xxix
Abstract	xxxiii
CHAPTER 1	1
Review of Literature	1
1.1 Introduction	1
1.1.1 Endocrine disrupting chemicals in environment: concept.....	1
1.1.2 Classes and sources of endocrine disrupting chemicals.....	1
1.2 Growing concern on endocrine disrupting compounds	7
1.2.1 Reproductive effects of estrogens in fish.....	8
1.2.2 Reproductive effects of anti-estrogens in fish.....	9
1.2.3 Reproductive effects of androgens in fish.....	10
1.2.4 Reproductive effects of anti-androgens in fish.....	11
1.3 Flutamide	13
1.3.1 Flutamide – nature and uses.....	13
1.3.2 Reproductive effects of flutamide.....	15
1.3.2.1 Mammalian studies.....	15
1.3.2.2 Fish studies.....	19
1.4 Phthalic acid esters	23
1.4.1 Phthalic acid esters – nature, uses and prevalence.....	23
1.4.2 Reproductive effects of phthalic acid esters.....	29
1.4.2.1 Mammalian studies.....	30
1.4.2.2 Fish studies.....	37
1.5 Gaps in Aquatic Ecotoxicology research	48
1.6 Hypotheses	52
1.6.1 Null hypotheses.....	52
1.6.2 Alternate hypotheses.....	53
7. Aims	54
8. References	57
CHAPTER 2	95
Effects of the commercial anti-androgen flutamide on the biomarkers of reproduction in male Murray rainbowfish (<i>Melanotaenia fluviatilis</i>)	

Authorship statement	96
Abstract	98
Introduction	98
Materials and methods	99
Culture and maintenance of fish.....	99
Fish exposure.....	99
Procedure for fish sampling.....	99
Chemical analyses of the water in testing tanks.....	100
Histological assessment of testicular tissues.....	100
Semi quantitative enzyme-linked immune-sorbent assay for VTG determination in plasma.....	100
Determination of the aromatase activity in the brain.....	100
Quantitative real-time polymerase chain reaction in liver samples.....	100
Data analyses.....	101
Results	101
Water quality and physical conditions during fish exposure.....	101
Water chemistry in the exposure tanks.....	101
Biological effects.....	102
Histological analyses of the testis.....	102
Hepatic expression of the genes for the receptors involved in steroidogenesis.....	102
Circulating levels of plasma vitellogenin protein.....	102
Aromatase activity in brain.....	102
Discussion	102
Conclusion	106
References	106

CHAPTER 3.....108

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

Authorship statement	109
Abstract	110
Introduction	110
Materials and methods	112
Test species.....	112
Test chemical.....	112
Test conditions.....	112
Sampling procedure.....	113
Chemical analysis of water.....	113
Histological analysis of ovarian tissue.....	113

Semi-quantitative vitellogenin analysis.....	113
Determination of brain aromatase activity.....	113
Steroid hormone measurement.....	114
Data analyses.....	114
Results	114
Water quality and physical conditions during fish exposure.....	114
Water chemistry of the exposure tanks.....	114
Biological effects.....	114
Histopathology of ovaries.....	114
Brain aromatase activity.....	115
Plasma vitellogenin concentration.....	115
Plasma sex steroid hormone concentrations.....	115
Discussion	116
Conclusion	117
References	117
CHAPTER 4	126
Effects of the model anti-androgen, flutamide on 17β-estradiol-induced hormonal imbalance in freshwater juvenile Murray rainbowfish (<i>Melanotaenia fluviatilis</i>)	
Authorship statement	127
Abstract	128
Introduction	130
Materials and methods	133
Culture and maintenance of fish.....	133
Chemicals.....	133
Fish exposure.....	134
Sampling procedure.....	134
Chemical analyses.....	135
Processing of tissues for histological investigation.....	137
Vitellogenin analysis.....	139
Measurement of steroid hormone concentrations.....	140
Data analyses.....	142
Results	142
Water chemistry of the exposure tanks.....	142
Water quality and physical conditions during fish exposure.....	142
Mortality and morpho-anatomical indices.....	143
Histopathological investigation of the gonads.....	143
Hormone concentrations.....	145
Vitellogenin concentration.....	145
Discussion	146

Conclusion	152
References	153
CHAPTER 5	179
Di-n-butyl phthalate causes estrogenic effects in adult male Murray rainbowfish (<i>Melanotaenia fluviatilis</i>)	
Authorship statement	180
Abstract	182
Introduction	182
Materials and methods	183
Culture and maintenance of fish.....	183
Fish exposure.....	183
Sampling procedure.....	184
Chemical analyses.....	184
Processing of tissues for histological examination.....	184
Semi-quantitative ELISA vitellogenin analysis.....	184
Evaluation of aromatase activity in the brain.....	185
RNA isolation and semi-quantitative RT-PCR in liver.....	185
Data analysis.....	185
Results	186
Water quality and physical conditions during fish exposure.....	186
Water chemistry during fish exposure.....	186
Biological effects.....	186
Testicular histopathology.....	186
Serum vitellogenin protein concentrations.....	187
Brain aromatase activity.....	188
Relative expression of genes involved in the expression of steroid receptors in the liver.....	188
Discussion	189
References	192
CHAPTER 6	195
Di-n-butyl phthalate causes anti-estrogenic effects in female Murray rainbowfish (<i>Melanotaenia fluviatilis</i>)	
Authorship statement	196
Abstract	198
Introduction	198
Materials and methods	199
Culture and maintenance of fish.....	199
Fish exposure.....	199

Sampling procedure.....	199
Chemical analyses.....	199
Processing of tissues for histological examination.....	200
Evaluating DnBP-induced histological changes.....	200
Semi-quantitative enzyme-linked immunosorbent assay VTG analysis.....	200
Data analyses.....	200
Results.....	200
Water quality during Murray rainbowfish exposure.....	200
Water chemistry.....	200
Biological effects.....	201
Ovarian histopathology.....	201
Serum VTG protein levels.....	202
Discussion.....	202
Conclusion.....	205
References.....	206
CHAPTER 7.....	208
Long-term exposures to di-n-butyl phthalate inhibit body growth and impair gonad development in juvenile Murray rainbowfish (<i>Melanotaenia fluviatilis</i>)	
Authorship statement.....	209
Abstract.....	211
Introduction.....	212
Materials and methods.....	215
Culture and maintenance of fish.....	215
Chemicals.....	215
Fish exposure.....	216
Sampling procedure.....	216
Water chemistry.....	217
Histological examination.....	218
Hormone analyses.....	220
Data analyses.....	221
Results.....	222
Water quality and physical conditions during the test.....	222
Analytical chemistry.....	222
Mortality and vital indices.....	223
Gonadal histopathology.....	223
Hormone analyses.....	224
Discussion.....	225
Conclusion.....	230
References.....	232

CHAPTER 8.....	253
Summary and conclusions	
8.1 Summary of the research.....	254
8.1.1 Effects of exposures to the pharmaceutical anti-androgen, flutamide on the biomarkers of reproduction in male, female and juvenile Murray rainbowfish.....	254
8.1.1.1 Testing null hypotheses.....	254
8.1.1.2 Summary of the results.....	255
8.1.1.3 Alternate hypotheses accepted.....	257
8.1.2 Effects of exposures to di-n-butyl phthalate on the biomarkers of reproduction in male, female and juvenile Murray rainbowfish.....	258
8.1.2.1 Testing null hypotheses.....	258
8.1.2.2 Summary of the results.....	259
8.1.2.3 Alternate hypotheses accepted.....	261
8.2 Conclusions.....	261
8.3 Recommendations for future.....	275
8.4 References.....	278

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 17 β -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

Table 1:	Some natural and synthetic chemicals detected in environment	3
Table 2:	Chemical structures of some of the commonly used non-steroidal anti-androgens	12
Table 3:	Some of the past literature on the effects of flutamide on the biomarkers of reproduction in mammals	16
Table 4:	Endocrine and reproductive effects of exposure to flutamide in fish from past research	19
Table 5:	Chemical structures of phthalic acid and the two most common environmental pollutants, di-butyl phthalate and diethylhexyl phthalate	22
Table 6:	Physical properties of various types of phthalates	23
Table 7:	Reported concentrations of various phthalates in aquatic environment worldwide	25
Table 8:	Some past research on the reproductive effects of phthalates in mammals	33
Table 9:	No observed effect concentrations, lowest observed effect concentrations and lethal concentration of selected phthalates in different classes of organisms	40
Table 10	Some of the past laboratory studies reporting reproductive, biochemical, histological, molecular and/or morphological effects of selected phthalates in fish	42

Chapter 2

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

Table 1:	Primer sequences (5'→3') of the genes for estrogen receptor α (<i>esr1</i>), estrogen receptor β (<i>esr2a</i>), vitellogenin (<i>vlg</i>), choriogenin L (<i>Chg-L</i>), androgen receptor α (<i>ARα</i>), androgen receptor β (<i>ARβ</i>), and 18S rRNA in Murray rainbowfish	96
----------	---	----

(Melanotaenia fluviatilis)

Table 2: Measurements (mean±standard error) of flutamide concentrations in fish tanks by high pressure liquid chromatography before the start of the exposure (day 0) and 24 h after spiking of the test solutions (days 1, 3, 5, and 7) and water quality parameters measured daily in fish tanks (n=2) 96

CHAPTER 3

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

Supplementary Endocrine and reproductive effects of exposure to flutamide in table 1: fish from selected past research

Supplementary Measured concentrations (mean ± standard error) of flutamide and table 2: water quality parameters (mean ± standard error) in the testing tanks. 127

Supplementary Condition factor, gonadosomatic index and hepatosomatic index table 3: (mean ± standard error) in Murray rainbowfish after exposure to 125 to 1000 µg/L flutamide for 7 days. 128

CHAPTER 4

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

Supplementary Measured concentrations (mean ± SE) of 17β-estradiol and table 1: 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). 174

CHAPTER 5

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

Table 1:	Primer sequences (5'→3') for oestrogen receptor α , oestrogen receptor β , vitellogenin, choriogenin L, androgen receptor α , androgen receptor β and 18S rRNA in Murray rainbowfish (<i>Melanotaenia fluviatilis</i>).	185
Table 2:	Measured concentrations of 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 3, 5 and 7 of the test (n = 2) and average water quality parameters (mean \pm SE) measured daily in fish tanks.	185
Table 3:	Changes in the condition factor, gonadosomatic index and hepatosomatic index in Murray rainbowfish (<i>Melanotaenia fluviatilis</i>) after exposure to DnBP for 7 days	186

CHAPTER 6

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

Table 1:	Measured concentrations (mean \pm standard error) of di-n-butyl phthalate in the Murray rainbowfish (<i>Melanotaenia fluviatilis</i>) testing tanks determined by GC-MS before the start (0 h; n = 2) and 24 h after of the start of the experiment (n = 6) and water quality parameters (mean \pm standard error) measured daily in the fish tanks (n = 14)	201
Table 2:	Changes in the condition factor, gonadosomatic index, and hepatosomatic index (mean \pm standard error) in Murray rainbowfish (<i>Melanotaenia fluviatilis</i>) after exposure to 125 μ g/L to 1000 μ g/L DnBP for 7 d	201
Table 3:	Changes in the height of granulosa cells (mean \pm standard error) and the thickness of the chorion (mean \pm standard error) in the	204

late vitellogenic follicles in Murray rainbowfish (*Melanotaenia fluviatilis*) after exposure to DnBP for 7 days

CHAPTER 7

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

Table 1:	Lowest observed effective concentrations (LOEC) of di-n-butyl phthalate that can cause physiological effects in juvenile Murray rainbowfish	240
Supplementary table 1:	Measured concentrations (mean \pm 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 \pm standard error) measured daily in fish tanks (n = 4).	252

LIST OF FIGURES

Chapter 1

Review of Literature

- Figure 1: Flow-chart depicting thesis structure 52

Chapter 2

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

- Figure 1: Changes in the condition factor (CF), gonadosomatic index (GSI), and hepatosomatic index (HSI) of male Murray rainbowfish (*Melanotaenia fluviatilis*) after treatment with flutamide. Lowercase letters indicate significant difference: a = significant difference from the water and solvent controls ($p \leq 0.05$); b = significant difference from the solvent control ($p \leq 0.05$). 97
- Figure 2: Photomicrographs of the testes of Murray rainbowfish (*Melanotaenia fluviatilis*) in the water control (A), showing spermatogonia (yellow arrow; inset), spermatocytes (black arrow), spermatids (green arrow), and sperms (blue arrow); and exposed to 1000 $\mu\text{g/L}$ flutamide (B), showing multinucleated cells (yellow arrow) and pyknotic cells (black arrow). In both (A) and (B), the solid line represents the height of the germinal epithelium. 98
- Figure 3: Photomicrographs of the testes of Murray rainbowfish (*Melanotaenia fluviatilis*) in the water control (A) exposed to 125 $\mu\text{g/L}$ flutamide (B), showing pyknotic cells (yellow arrow) and interstitial fibrosis (black arrow); exposed to 250 $\mu\text{g/L}$ flutamide (C), showing decrease in the relative proportion of sperms and increase in the proportion of spermatocytes and spermatids; and exposed to 500 $\mu\text{g/L}$ flutamide (D), showing interstitial fibrosis (black arrow). 98
- Figure 4: Relative abundance of the transcripts for estrogen receptor α (*esr1*), estrogen receptor β (*esr2a*), vitellogenin (*vtg*), choriogenin L receptor 99

(*chg-L*), androgen receptor α (*ARA*), and androgen receptor β (*Ar β*) in the liver of adult male Murray rainbowfish (*Melanotaenia fluviatilis*) after exposure to 125 $\mu\text{g/L}$, 250 $\mu\text{g/L}$, 500 $\mu\text{g/L}$, and 1000 $\mu\text{g/L}$ flutamide. Data normalized to 18S rRNA housekeeping gene. Lowercase letters indicate significant difference: a = significant difference from the corresponding water and solvent controls ($p \leq 0.05$); b = significant difference from the corresponding 125 $\mu\text{g/L}$ flutamide treatment ($p \leq 0.05$).

- Figure 5: Relative changes in the levels of vitellogenin protein (arbitrary units per mg protein) in the plasma of adult male Murray rainbowfish (*Melanotaenia fluviatilis*) after exposures to 125 $\mu\text{g/L}$, 250 $\mu\text{g/L}$, 500 $\mu\text{g/L}$, and 1000 $\mu\text{g/L}$ flutamide for 7 d. Lowercase letters indicate significant difference: a = significant difference from the water control, solvent control, 125 $\mu\text{g/L}$ flutamide, and 250 $\mu\text{g/L}$ flutamide treatments ($p \leq 0.05$); b = significant difference from the 500 $\mu\text{g/L}$ flutamide treatment ($p \leq 0.05$). 99
- Figure 6: Activity of brain aromatase (fmol/h/mg protein) in adult male Murray rainbowfish (*Melanotaenia fluviatilis*) exposed to 125 $\mu\text{g/L}$, 250 $\mu\text{g/L}$, 500 $\mu\text{g/L}$, and 1000 $\mu\text{g/L}$ flutamide for 7 d. a = significant difference from the control and the solvent control ($p \leq 0.05$). 99

CHAPTER 3









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

- Figure 1: Transverse section of the ovary of Murray rainbowfish in (A) control (B) 125 $\mu\text{g/L}$ flutamide and (C) 1000 $\mu\text{g/L}$ flutamide treatments. Note the compact arrangement of oocytes in control, uncondensed arrangement in 125 $\mu\text{g/L}$ treatments and interstitial fibrosis (←■) in 1000 $\mu\text{g/L}$ treatment. Perinucleolar oocyte (←■); cortical alveolar oocyte (←■); early vitellogenic oocyte (←■); late vitellogenic oocyte (←■); atretic (←■). 129
- Figure 2: Comparison in the proportions of the oocytes in various stages of 130

	development in the ovaries of Murray rainbowfish in the control, and 125-1000 $\mu\text{g/L}$ flutamide treatments. No spawning oocytes were detected in the ovaries of 250-1000 $\mu\text{g/L}$ flutamide treated fish.	
Figure 3:	Comparison in the sizes of the oocytes in various stages of development in the ovaries of Murray rainbowfish in the control, and 125-1000 $\mu\text{g/L}$ flutamide treatments (^a : Significant difference from the corresponding control [$p \leq 0.05$]).	131
Figure 4:	Brain aromatase activity of Murray rainbowfish in the control, and 125-1000 $\mu\text{g/L}$ flutamide treatments (^a : Differs significantly from the corresponding value of the control [$p \leq 0.05$]).	132
Figure 5:	Circulating levels of plasma vitellogenin in Murray rainbowfish in the control, and 125-1000 $\mu\text{g/L}$ flutamide treatments (^a : Differs significantly from the corresponding value of the control [$p \leq 0.05$]).	133
Figure 6:	(A) Circulating levels of plasma 17 β -estradiol and 11-keto testosterone in Murray rainbowfish in the control and 125-1000 $\mu\text{g/L}$ flutamide treatments. (B) Ratio of the concentrations of 17 β -estradiol and 11-keto testosterone in Murray rainbowfish in the control and 125-1000 $\mu\text{g/L}$ flutamide treatments (^a : Differs significantly from the corresponding value of the control [$p \leq 0.05$]).	134
Supplementary	Correlation connection diagram depicting strong positive correlations	135
Figure 1:	(solid lines) between experimental variables. E2 and VTG $r = 0.926$, $p = 0.008$; E2 and aromatase $r = 0.987$, $p = 0.0002$; VTG and aromatase $r = 0.950$, $p = 0.004$; VTG and 11-KT $r = 0.902$, $p = 0.014$; E2 and the size of perinucleolar oocytes $r = 0.839$; $p = 0.037$ E2 – 17 β -estradiol in plasma, VTG – vitellogenin in plasma, 11-KT – 11-ketotestosterone in plasma r - Pearson correlation coefficient	

CHAPTER 4

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

- 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 17 β -estradiol for 35 days. Bars represent standard error. Means not followed by the same letter are significantly different ($p \leq 0.05$) 175
- 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 μ g/L flutamide (Flu high) D) 25 ng/L E2 + 250 μ g/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. 176
- 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 μ g/L flutamide (Flu high) D) 25 ng/L E2 + 250 μ g/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 177
- 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 μ g/L flutamide (Flu high) and B) 25 ng/L E2 + 250 μ g/L flutamide 178

(E2+Flu high). The arrows represent perinucleolar oocytes oocytes (◀) and early vitellogenic oocytes (◀). Note the development of spermatocytes (◀) between the oocytes.

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.

179

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.

180

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

Supplementary figure 1: 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*)

Figure 1: Photomicrographs of the testes of Murray rainbowfish (*Melanotaenia fluviatilis*). (A and D) Water control, (B and E) 500 µg/L DnBP-exposed, (C and F) 1000 µg/L DnBP exposed.

186

◀ Represents spermatogonia, ◀ represents spermatocytes,

← represents spermatids and ← represents spermatozoa. Note the increase in spermatogonial numbers in the testes of the fish exposed to 500 µg/L 1000 µg/L of DnBP. The testicular sections within the same treatment shown here are from different fish.

Figure 2: Photomicrographs of the testes of Murray rainbowfish (*Melanotaenia fluviatilis*). (A) Water control and (B–D) exposed to 1000 µg/L DnBP. 187

← Represents spermatogonia, ← represents spermatocytes, ← represents spermatids and ← represents spermatozoa. Note the development of vacuolation (⚡), interstitial fibrosis (⚡), apoptotic cells (⚡) and increased proportion of spermatogonia (←) in the testes of DnBP treated fish.

Figure 3: Changes in the sizes of the germ cells (spermatogonia, spermatocyte A, spermatocyte B and spermatids) in the testes of adult male Murray rainbowfish (*Melanotaenia fluviatilis*) after exposures to DnBP for 7 days. Means not followed by the same uppercase letter are significantly different ($p \leq 0.05$). Bars depict standard error. Two replicates containing four fish each were used. 187

Figure 4: Changes in the proportions of the germ cells (spermatogonia, spermatocytes, spermatids and spermatozoa) in the testes of adult male Murray rainbowfish (*Melanotaenia fluviatilis*) after exposures to DnBP for 7 days. Means not followed by the same uppercase letter are significantly different ($p \leq 0.05$). Bars depict standard error. Two replicates containing four fish each were used. 188

Figure 5: Changes in serum vitellogenin levels in adult male rainbowfish (*Melanotaenia fluviatilis*) after exposures to DnBP for 7 days. Means not followed by the same uppercase letter are significantly different ($p \leq 0.05$). Bars depict standard error. Two replicates containing four fish each were used. 188

Figure 6: Changes in brain aromatase activity adult male rainbowfish (*Melanotaenia fluviatilis*) exposed to DnBP for 7 days. Means not followed by the same uppercase letter are significantly different ($p \leq 0.05$). Bars depict standard error. Two replicates containing four fish 188

each were used.

- Figure 7: Relative abundance of the transcripts for (A) oestrogen receptor α ($Er\alpha$), (B) $Er\beta$, (C) vitellogenin (VTG), (D) choriogenin (ChgL), (E) androgen receptor α ($Ar\alpha$) and (F) $Ar\beta$ in the liver of adult male Murray rainbowfish (*Melanotaenia fluviatilis*) after exposures to DnBP for 7 days. Data normalised to 18S rRNA housekeeping gene. Means not followed by the same uppercase letter are significantly different ($p \leq 0.05$). Bars depict standard error. Two replicates containing four fish each were used. 189

CHAPTER 6

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

- Figure 1: Hematoxylin and eosin–stained vertical sections of the ovaries of Murray rainbowfish (*Melanotaenia fluviatilis*). (A) Control, (B) 125 $\mu\text{g/L}$ di-n-butyl phthalate (DnBP), (C) 250 $\mu\text{g/L}$ DnBP. Scale bar = 500 μm for all photomicrographs. (⇒ Perinucleolar oocytes; ⇒ cortical alveolar oocytes; ⇒ early vitellogenic oocytes; ⇒ late vitellogenic oocytes; ⇒ spawning oocytes; ★ atretic oocytes.) 201
- Figure 2: Variation in the proportion of the different developmental stages of the oocytes in Murray rainbowfish (*Melanotaenia fluviatilis*) after exposure to 125 $\mu\text{g/L}$ to 1000 $\mu\text{g/L}$ di-n-butyl phthalate (DnBP) for 7 days. 202
- Figure 3: Developmental anomalies in the ovaries of adult Murray rainbowfish (*Melanotaenia fluviatilis*) after treatment with 1000 $\mu\text{g/L}$ di-n-butyl phthalate (DnBP) for 7 d. ★ Represents (A) impaired yolk production (scale bar = 2 mm), (B) granulomatous inflammation (scale bar = 100 μm), (C) cyst (scale bar = 200 μm), (D) interstitial fibrosis (scale bar = 500 μm), (E) shrunken ooplasm (scale bar = 500 μm), and (F) granulosa cell hyperplasia (scale bar = 100 μm). 203
- Figure 4: Variation in the sizes (mean standard error) of the different developmental stages of the oocytes in Murray rainbowfish 204

(*Melanotaenia fluviatilis*) after exposure to di-n-butyl phthalate (DnBP) for 7 d.

- Figure 5: Variation (mean \pm standard error) in the circulating levels of plasma vitellogenin in Murray rainbowfish (*Melanotaenia fluviatilis*) after a subacute exposure to di-n-butyl phthalate (DnBP) for 7 d. 205

CHAPTER 7

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

- Figure 1: Figure 1: (A) Lengths (B) Weights and (C) Condition factors of juvenile Murray rainbowfish after exposures to 5, 15 and 50 $\mu\text{g/L}$ 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). 241

- Figure 2: 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) 242

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

- Figure 3: Figure 3: Photomicrographs of the vertical sections of the abdominal region of juvenile Murray rainbowfish from the control group showing 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). 244

T: Testis, SG: Spermatogonia, SC: Spermatocytes, SZ: Spermatozoa

- Figure 4: Figure 4: Photomicrographs of the vertical sections of the abdominal region of juvenile Murray rainbowfish showing the gonads after exposures to (A) 5 $\mu\text{g/L}$ di-n-butyl phthalate (DnBP) for 60 days (Stage 0 – immature); (B) 50 $\mu\text{g/L}$ DnBP for 30 days (Stage 0 – 246

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

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).	248
Supplementary Figure 6:	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.	251

CHAPTER 8

Summary and conclusions

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	263
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. Solid blue arrows are the data from the present study. E2: 17β-estradiol; 11-KT: 11-keto testosterone; VTG: Vitellogenin	264
Figure 3:	Hypothetical model of biological effects of flutamide and/or E2 in juvenile male Murray rainbowfish. Green arrows represent up-regulation and red arrows represent down-regulation based on the	266

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 and/or E2 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. 267

Figure 5: Hypothetical model of biological effects of DnBP 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. 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 269

Figure 6: Hypothetical model of biological effects of DnBP in adult female 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. DnBP: Di-n-butyl phthalate; E2: 17β-Estradiol; VTG: Vitellogenin; 11-KT: 11-Ketosterone 270

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: 272

Di-n-butyl phthalate; E2:17 β -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	17 β -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-pituitary-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 phthalate
MEHHP	Mono-(2-ethyl-5-hydroxyhexyl) phthalate
MEOHP	Mono-(2-ethyl-5-oxohexyl) phthalate
M1	2-[(3,5-dichlorophenyl)-carbonyl]oxy-2-methyl-3-butenoic acid
M2	3',5'-dichloro-2-hydroxy-2-methylbut-3-enamide
MT	17 α -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
3 β -HSD	3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase
11 β -hsd2	11 β -hydroxysteroid dehydrogenase 2
11-KT	11-keto testosterone
17 β -hsd12	17 β -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 17 β -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 17 β -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 17 β -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 µg/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 (AR α) and AR β . The expression levels of the genes for the estrogen receptor α (ER α) 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 ER α 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 $< 9.9 \mu\text{g/L}$ 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.