CHRONIC PHARMACEUTICAL EXPOSURE IMPACTS ON ZEBRAFISH

# CHRONIC EXPOSURE TO ENVIRONMENTALLY RELEVANT PHARMACEUTICAL CONCENTRATIONS EFFECTS REPRODUCTIVE AND DEVELOPMENTAL PHYSIOLOGY IN ZEBRAFISH (*Danio rerio*)

By:

MICHAL GALUS, H.B.Sc

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

© Copyright by Michal Galus, July 2014, Doctor of Philosophy

Doctor of Philosophy (2014)
Department of Biology

McMaster University
Hamilton, Ontario

TITLE: Chronic Exposure to Environmentally Relevant Pharmaceutical Concentrations Effects Reproductive and Developmental Physiology in Zebrafsih (*Danio rerio*)

AUTHOR: Michal Galus, H.B.Sc. (McMaster University)

SUPERVISOR: Dr. Joanna Y. Wilson

NUMBER OF PAGES: xxi, 266

## ABSTRACT

The presence of pharmaceuticals and personal care products (PPCPs) in the aquatic environment has been a growing issue of concern over the past twenty years. Compounds from various pharmaceutical classes have been detected at ng to µg $L^{-1}$ concentrations in waste water effluent, surface, ground and drinking water. Although the concentrations required for these compounds to elicit a therapeutic response is higher than what is detected in the aquatic environment, the impacts pharmaceuticals may have on aquatic species under chronic or mixture conditions remains largely unknown. This thesis addresses this knowledge gap by evaluating the impacts of chronic exposure to four frequently detected pharmaceuticals and pharmaceutical mixtures on the physiology of the model teleost, zebrafish (*Danio rerio*).

Environmentally relevant concentrations of acetaminophen, carbamazepine, gemfibrozil and venlafaxine significantly reduced zebrafish fecundity and increased embryonic mortality. Pharmaceutical exposure to gemfibrozil and carbamazepine altered the structural morphology of the ovary; all compounds tested altered kidney histology. As exposure in the environment is rarely ever to a single compound, animals were exposed to a quaternary mixture of the four compounds and wastewater effluent. Under mixture conditions, reproductive, developmental and histological effects were also observed, however they generally were more severe then those seen with single compound exposure. Overall, these results showed that chronic, low dose pharmaceutical exposure were sufficient to induce a suite of physiological effects suggesting an overall decrease in fish health.

Effects on offspring after chronic parental exposure to gemfibrozil and carbamazepine resulted in alterations in male breeding behaviour, reduced fecundity, decreased sperm velocity and induced morphological changes to spermatozoa. These novel findings expand the limited knowledge base of studies examining effects on offspring. Lastly, acetaminophen was confirmed to elicit its developmental impacts in fish via the cyclooxygenase pathway; the same mechanism of action as observed in mammals. This thesis has made significant contributions to identifying the physiological consequences of environmental pharmaceutical toxicity to fish.

## ACKNOWLEDGEMENTS

First and foremost I would like to thank my parents; Stanislaw and Edwarda Galus. Both of you have taught me the meaning of the word sacrifice and if it was not for your selfless actions, none of my accomplishments would have been possible. Both of you have been the most tolerating, loving and understanding people throughout my academic career and words do not do justice to how much I love you. Thank you from the deepest part of my heart for helping shape my future. I owe all my success to you. To my sister; Justyna Galus, thank you for keeping me grounded and always being there for me regardless of my stress level. I know I can always count on you in spite of what life throws at me.

Thank you to my extended Wilson Lab family for all the coffee breaks, laughs and good times. Through your teasing, joking and high spirits, you all made being in the lab a fun experience. A special thank you to Dr. Derek Alsop who is the epitome of post-doctoral fellow. Regardless of his work load, Derek was always willing to stop what he was doing and answer my barrage of questions or offer great advice to keep the project going. His knowledge of current memes always made for a good time in the lab. (╯°□°)╯︵ ┻━┻ I would also like to extend a special thank you to Lana Shaya; your advice, willingness to edit, grammar lessons and encouragement during the writing process have been invaluable.

Thank you to my committee members, Dr. Grant McClelland and Dr. Michael O`Donnell for your excellent scientific advice. Both of you were always willing to offer guidance and encouragement along the way and your scientific expertise helped polish my project.

Last, but certainly not least, I would like to extend my deepest and most sincere gratitude to my supervisor Dr. Joanna Y. Wilson. I could have not asked for a better mentor. You were always there and always willing to discuss my latest discovery, offer encouragement when the science was not working, provide clarity to unknown techniques or somehow put a positive spin on carrying out 24 hour experiments. Through your hands

off approach, I was able to constantly stay motivated and engaged in my research. You encouraged me to follow the science and find the answers to the questions I was most interested in; this I find exceptional. I will forever be grateful for everything you have done for me. You were instrumental in shaping my research career and I look forward to the day where I can call you my colleague. Thank you for everything.

After 5 years, countless gallons of coffee and many sleepless nights, it is finally over. If you are still with me, block off a few hours and enjoy the read. Achievement Unlocked – Ph.D. Earned. GOAL!!

# TABLE OF CONTENTS

| | |
|---|---|
| ABSTRACT | iii |
| ACKNOWLEDGEMENTS | iv |
| TABLE OF CONTENTS | vi |
| LIST OF TABLES | xii |
| LIST OF FIGURES | xiv |
| LIST OF ABBREVIATIONS | xvii |
| DECLARATION OF ACADEMIC ACHIEVEMENT | xx |

**CHAPTER 1**
**GENERAL INTRODUCTION**

**PHARMACEUTICALS IN THE ENVIRONMENT** .......... 1
**BIOLOGICAL IMPACTS OF PHARMACEUTICALS IN FISH SPECIES** .......... 4
*Analgesics and Anti-Inflammatories* .......... 4
*Antibiotics* .......... 6
*Anti-Depressants* .......... 7
*Anti-Epileptics and Mood Stabilizers* .......... 10
*Beta Blockers* .......... 11
*Lipid Regulators* .......... 12
**ZEBRAFISH AS A MODEL ORGANISIM** .......... 14
**THESIS GOALS** .......... 17
**CHAPTER SUMMARY** .......... 19
**REFERENCES** .......... 22

**CHAPTER 2**

**CHRONIC, LOW CONCENTRATION EXPOSURE TO PHARMACEUTICALS IMPACTS MULTIPLE ORGAN SYSTEMS IN ZEBRAFISH (*Danio rerio*)**

**ABSTRACT** .......... 34
**INTRODUCTION** .......... 35
**METHODS** .......... 38
*Fish Care* .......... 38
*Compounds Tested* .......... 38
*Adult Zebrafish Exposures* .......... 39
*Embryonic Zebrafish Exposures* .......... 39
*Water Sample Analyses* .......... 40
*Reproductive and Developmental Endpoints* .......... 42
*Blood and Tissue Sampling* .......... 43
*Histology* .......... 44
*TUNEL Assay* .......... 45
*Sex Steroid Hormones* .......... 46
*Statistical Analysis* .......... 46
**RESULTS** .......... 47
*Waterborne Pharmaceutical Concentrations* .......... 47
*Pharmaceutical impacts on reproduction* .......... 47
*Pharmaceutical impacts on development* .......... 48
*Organ Histology and TUNEL staining* .......... 49
*Sex Steroid Concentrations* .......... 51
**DISCUSSION** .......... 51
*Reproductive Endpoints* .......... 53
*Kidney Histology* .......... 56
*Embryonic Effects* .......... 57
*Implications for environmental health* .......... 57
**ACKNOWLEDGEMENTS** .......... 58
**REFERENCES** .......... 75

**CHAPTER 3**

**CHRONIC EFFECTS OF EXPOSURE TO A PHARMACEUTICAL MIXTURE AND MUNICIPAL WASTEWATER IN ZEBRAFISH**

**ABSTRACT** .......... 80
**INTRODUCTION** .......... 81
**METHODS** .......... 85
*Wastewater* .......... 85
*Test Chemicals* .......... 85
*Exposures* .......... 86
*Tank Water Samples for PPCP Analyses* .......... 89
*Wastewater Samples for PPCP Analyses* .......... 90
*PPCP Analyses in Extracted Tank Water and Effluent* .......... 91
*Reproductive and Developmental Endpoints* .......... 92
*Blood and Tissue Sampling* .......... 93
*Histology* .......... 93
*TUNEL Assay* .......... 94
*Plasma Hormones* .......... 95
*Statistical Analysis* .......... 95
**RESULTS** .......... 97
*PPCPs in Wastewater* .......... 97
*Measured Concentrations* .......... 98
*Embryo production* .......... 99
*Effects on Embryos* .......... 100
*Histology and TUNEL Staining* .......... 101
*Sex Steroid Concentrations* .......... 102
**DISCUSSION** .......... 102
*Reproductive Endpoints* .......... 105

*Embryonic Exposures* .......... 106
*Histology* .......... 107
*Mixture Toxicity* .......... 108
**ACKNOWLEDGEMENTS** .......... 111
**REFERENCES** .......... 122

---

**CHAPTER 4**

**EFFECTS OF CHRONIC, PARENTAL PHARMACEUTICAL EXPOSURE ON ZEBRAFISH (*Danio rerio*) OFFSPRING**

**ABSTRACT** .......... 127
**INTRODUCTION** .......... 128
**METHODS** .......... 133
*Compounds Tested* .......... 133
*Adult Exposures* .......... 133
*Pairwise and Reciprocal Breeding* .......... 135
*Courtship Behaviour* .......... 136
*Sperm Collection, Velocity and Morphology* .......... 137
*Histology* .......... 139
*Statistics* .......... 140
**RESULTS** .......... 141
*Embryo Production during Pairwise & Reciprocal Crosses* .......... 141
*Courtship Behaviours* .......... 142
*Sperm Velocity & Morphology* .......... 143
*Histology* .......... 144
**DISCUSSION** .......... 144
*Breeding Success & Embryo Production* .......... 147
*Courtship Behaviour* .......... 150
*Sperm Morphology & Velocity* .......... 153

*Conclusion* ........ 156
**ACKNOWLEDGEMENTS** ........ 157
**REFERENCES** ........ 176

---

**CHAPTER 5**

**PROSTAGLANDINS PREVENT ACETAMINOPHEN INDUCED EMBRYO TOXICITY IN ZEBRAFISH (*Danio rerio*)**

**ABSTRACT** ........ 183
**INTRODUCTION** ........ 184
**METHODS** ........ 187
*Fish Care* ........ 187
*Compounds Tested* ........ 188
*Experimental Design* ........ 188
*Acetaminophen Dose Response* ........ 189
*Acetaminophen Time Course* ........ 189
*Total COX Activity* ........ 189
*COX Gene Expression* ........ 190
*Prostaglandin E2 Supplementation* ........ 192
*Statistics* ........ 192
**RESULTS** ........ 193
*Acetaminophen Dose Response Curve* ........ 193
*Acetaminophen Time Course* ........ 193
*Total COX Activity* ........ 194
*Gene Expression* ........ 195
*Prostaglandin E2 Supplementation* ........ 196
**DISCUSSION** ........ 196
*Acetaminophen Dose Response* ........ 197
*Acetaminophen Time Course* ........ 198

*Cyclooxygenase Activity and Gene Expression* ... 198
*Does ACE cause embryo toxicity via inhibition of COX activity?* ... 200
*Conclusion* ... 201
**ACKNOWLEDGEMENTS** ... 202
**REFERENCES** ... 221

---

**CHAPTER 6**

**GENERAL SUMMARY AND CONCLUSIONS**

**GENERAL SUMMARY AND CONCLUSIONS** ... 225
*Compound and Concentration Justification* ... 225
*Acute versus Chronic Toxicity* ... 227
*Sex Specific Effects on Reproduction* ... 229
*Common Endpoints of Pharmaceutical Toxicity* ... 232
*Mixture Toxicity* ... 235
**CONCLUSIONS** ... 237
**FUTURE DIRECTIONS** ... 238
**REFERENCES** ... 240

---

**APPENDIX** ... 247

---

## LIST OF TABLES

**Table 2.1**

Reproduction in zebrafish exposed to pharmaceuticals ........................................ 58

**Table 4.1**

Pictogram describing adult courtship behaviours amongst zebrafish ............................ 158

**Table 4.2**

Mean clutch size in F1 zebrafish during pairwise and reciprocal matings ..................... 160

**Table 4.3**

Differences in duration and frequency of breeding behaviours within CTL pairs ......... 162

**Table 5.1**

Embryonic COX activity in zebrafish during development ........................................... 203

**Supplementary Table S1**

Measured aqueous pharmaceutical concentration.......................................................... 248

**Supplementary Table S2**

Histopathological damage in the kidney of zebrafish exposed to pharmaceuticals ........ 250

**Supplementary Table S3**

Histopathological damage in the liver of zebrafish exposed to pharmaceuticals ........... 252

**Supplementary Table S4**

Histopathological damage in the gonads of zebrafish exposed to pharmaceuticals ....... 254

**Supplementary Table S5**

Mean concentrations (µg $L^{-1}$; N=3) of selected pharmaceutical compounds in triplicate samples collected from a WWTP............................................................................ 256

**Supplementary Table S6**

Mean (±SD) measured aqueous concentrations (µg $L^{-1}$; N=3) of acetaminophen (ACE), carbamazepine (CBZ), gemfibrozil (GEM) and venlafaxine (VEN) in samples of tank water collected during exposures of zebrafish to mixtures (MIX) and treated wastewater (WWE)................................................................................................................. 258

**Supplementary Table S7**

Incidence and severity of histopathological damage in the kidney of zebrafish exposed to a mixture of pharmaceuticals (MIX) and wastewater effluent (WWE)........................... 260

**Supplementary Table S8**

Incidence and severity of histopathological damage in the ovaries of female zebrafish exposed to a mixture of pharmaceuticals (MIX) and wastewater effluent (WWE).........262

## LIST OF FIGURES

**Figure 2.1**

A schematic of the experimental design and timing of sampling .................................... 61

**Figure 2.2**

Cumulative daily embryo production in zebrafish chronically exposed to pharmaceuticals ........................................................................................................... 63

**Figure 2.3**

Total mortality and developmental abnormalities in zebrafish after embryonic exposure to pharmaceuticals ........................................................................................................... 65

**Figure 2.4**

Histological alterations in kidney after pharmaceutical exposure ................................... 67

**Figure 2.5**

Histological alterations in liver and ovary after pharmaceutical exposure ....................... 69

**Figure 2.6**

Apoptosis within the oocyte after pharmaceutical exposure ........................................... 71

**Figure 2.7**

Plasma concentrations of estradiol and 11-keto testosterone after pharmaceutical exposure ........................................................................................................................... 73

**Figure 3.1**

Cumulative daily embryo production from zebrafish in tests with breeding fish chronically exposed to a pharmaceutical mixture (A) and wastewater effluent (B ) ....... 112

**Figure 3.2**

Mean (N=3) percent total mortalities (A) and developmental abnormalities (B) in zebrafish embryos after exposure to a pharmaceutical mixture (MIX) and to wastewater effluent (WWE) ........................................................................................................ 114

**Figure 3.3**

Histology of H&E stained sections (60 x) of the kidney from adult zebrafish from control and exposed treatments ........................................................................................ 116

**Figure 3.4**

Histology of H&E stained sections (20 x) of the ovaries from adult female zebrafish from the control treatment and the treatment with exposure to wastewater effluent (WWE) .. 118

**Figure 3.5**

Apoptosis within the oocyte after pharmaceutical exposure ........................................ 120

**Figure 4.1**

Breeding success in $F_1$ zebrafish during pairwise and reciprocal matings ..................... 164

**Figure 4.2**

Mean embryo production in $F_1$ zebrafish during within treatment and reciprocal matings ........................................................................................................... 166

**Figure 4.3**

Duration of male courtship behaviours during $F_1$ mating ............................................. 168

**Figure 4.4**

Frequency of male Courtship behaviours during $F_1$ mating .......................................... 170

**Figure 4.5**

Sperm velocity in male $F_1$ zebrafish ................................................................. 172

**Figure 4.6**

Sperm Morphology in male $F_1$ zebrafish ........................................................... 174

**Figure 5.1**

Dose response relationship for abnormalities in zebrafish after embryonic exposure to acetaminophen ..................................................................................................... 205

**Figure 5.2**

Dose response relationship for mortality in zebrafish after embryonic exposure to acetaminophen. .................................................................................................... 207

**Figure 5.3**

Time course for abnormalities during zebrafish development with embryonic exposure to acetaminophen ..................................................................................................... 209

**Figure 5.4**

Time course for mortality during zebrafish development with embryonic exposure to acetaminophen ..................................................................................................... 211

**Figure 5.5**

Total cyclooxygenase activity in developing zebrafish with embryonic exposure to acetaminophen ..................................................................................................... 213

**Figure 5.6**

mRNA expression of cox-1, 2a and 2b in embryonic zebrafish after exposure to 10 nM 6-Formylindolo(3,2-b)carbazole (FICZ)........215

**Figure 5.7**

Total abnormalities in developing zebrafish with embryonic exposure to acetaminophen alone and co-exposed to prostaglandin E2........217

**Figure 5.8**

Mortality in zebrafish with embryonic exposure to acetaminophen alone and co-exposed with prostaglandin E2........219

**Supplementary Figure S1**

Mean (N=3) plasma concentrations of estradiol and 11-ketotestosterone in adult male and female zebrafish after 6 weeks exposure to a pharmaceutical mix (MIX) and to wastewater effluent (WWE)........264

## LIST OF ABBREVIATIONS

| | |
|---|---|
| °C | Degree Celsius |
| μg $L^{-1}$ | Microgram per Litre |
| μm | Micrometers |
| μM | Micromoles |
| μS | Microsiemens |
| ANOVA | Analysis of Variance |
| 11-KT | 11-Keto Testosterone |
| AA | Arachidonic Acid |
| ACE | Acetaminophen |
| AhR | Aryl Hydrocarbon Receptor |
| APCI | Atmospheric Pressure Chemical Ionization |
| BPA | Bisphenol A |
| BM | Basement Membrane |
| $CaCl_2$ | Calcium Chloride |
| CBZ | Carbamazepine |
| COX | Cyclooxygenase |
| CTL/CTRL | Control |
| DAB | Diaminobenzidine |
| DMSO | Dimethyl Sulfoxide |
| DuP-697 | 5-Bromo-2-(4-fluorophenyl)-3-(4-methylsulfonyl)phenyl-thiophene |
| $E_2$ | Estradiol |
| EDC | Endocrine Disrupting Chemicals |
| ESI | Electrospray Ionization |

| | |
|---|---|
| $F_0$ | Parental Generation |
| $F_1$ | First Filial Generation |
| FICZ | 6-Formylindolol[3,2-b]carbazole |
| G | Granulosa Cell Layer |
| GABA | γ-aminobutyric acid |
| GEM | Gemfibrozil |
| $H_2O$ | Water |
| $H_2SO_4$ | Sulphuric Acid |
| HPF | Hours Post Fertilization |
| HPLC | High Performance Liquid Chromatography |
| KCl | Potassium Chloride |
| LCA | Life Cycle Assessment |
| L | Litres |
| LOQ | Limits of Quantitation |
| LC-MS/MS | Liquid Chromatography and Tandem Mass Spectrometry |
| M | Molar |
| MeOH | Methanol |
| $MgSO_4$ | Magnesium Sulphate |
| MIX | Mixture |
| mg | Milligrams |
| mL | Millilitres |
| NaCL | Sodium Chloride |
| NaOH | Sodium Hydroxide |
| NOEC | No Observed Effect Concentration |
| ng | Nanogram |
| $NH_4OH$ | Ammonium Hydroxide |

| | |
|---|---|
| NSAID | Non-steroidal anti-inflammatory |
| PBS | Phosphate Buffered Saline |
| PCBs | Polychlorinated Biphenyls |
| PG | Prostaglandin |
| pH | Measure of Acidity or Basicity /-log[$H^+$] |
| POD | Peroxidase |
| PPARα | Peroxisomal Proliferator-activated Receptor Alpha |
| PPCP | Pharmaceuticals and Personal Care Products |
| RT-PCR | Reverse Transcriptase Polymerase Chain Reaction |
| S1 | Early Vitellogenic Oocytes |
| S III-IV | Stage 3 or 4 Mature Ooytes |
| Sc-560 | 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl-pyrazole |
| SEM | Standard Error of the Mean |
| SNRI | Selective Norepinephrine Reuptake Inhibitor |
| SPE | Solid Phase Extraction |
| SSRI | Selective Serotonin Reuptake Inhibitor |
| SD/STDEV | Standard Deviation |
| TUNEL | dUTP Nick End Labeling |
| *Vap* | Smooth Path Velocity |
| V | Ooplasmic Vesicles |
| VEN | Venlafaxine |
| WWE | Wastewater Effluent |
| WWTP | Wastewater Treatment Plant |
| Zr | Zona Radiata |

## DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis is organized in a sandwich format approved by McMaster University and with the recommendation of the supervisory committee. The thesis consists of six chapters. Chapter 1 comprises an introduction and summary of the literature on pharmaceutical toxicity. Chapters 2-5 comprise discrete manuscripts published (Chapters 2-4) or submitted (Chapter 5) to peer-reviewed scientific journals. Finally, Chapter 6 summarizes the consequences of environmental pharmaceutical exposure and establishes directions for future research.

| | |
|---|---|
| **CHAPTER 1:** | **GENERAL INTRODUCTION** |
| **CHAPTER 2:** | **CHRONIC, LOW CONCENTRATION EXPOSURE TO PHARMACEUTICALS IMPACTS MULTIPLE ORGAN SYSTEMS IN ZEBRAFISH (*Danio rerio*)** |
| Authors: | Michal Galus, Nina Kirischian, Sarah Higgins, James Purdy, Justin Chow, Sahaana Rangaranjan, Hongxia Li, Chris Metcalfe and Joanna Y. Wilson. |
| Date Accepted: | December 13, 2012 |
| Journal: | Aquatic Toxicology |
| Comments: | M.G. conducted this study and wrote the manuscript under the supervision of J.Y.W. N.K., S.H., J.P., J.C., and S.R. assisted with animal husbandry, experimental exposures and tissue collection. S.H., J.P., J.C., and S.R. were involved in determining the effects on developing embryos. H.L. and C.M. provided analytical support. All authors provided editoral comments. |
| **CHAPTER 3:** | **CHRONIC EFFECTS OF EXPOSURE TO A PHARMACEUTICAL MIXTURE AND MUNICIPAL WASTEWATER IN ZEBRAFISH** |
| Authors: | Michal Galus, Judy Jeyaranjaan, Emily Smith, Hongxia Li, Chris Metcalfe and Joanna Y. Wilson. |
| Date Accepted: | December 13, 2012 |
| Journal: | Aquatic Toxicology |

Comments: M.G. conducted this study and wrote the manuscript under the supervision of J.Y.W. J.J. and E.S. assisted with animal husbandry, experimental exposures and tissue collection. J.J. was involved in determining the effects on developing embryos. H.L. and C.M. provided analytical support. All authors provided editorial comments.

**CHAPTER 4: EFFECTS OF CHRONIC, PARENTAL PHARMACEUTICAL EXPOSURE ON ZEBRAFISH (*Danio rerio*) OFFSPRING**

Authors: Michal Galus, Sahaana Rangaranjan, Anderson Lai, Lana Shaya, Sigal Balshine and Joanna Y. Wilson.

Date Accepted: January 18, 2014

Journal: Aquatic Toxicology

Comments: M.G. conducted this study and wrote the manuscript under the supervision of J.Y.W. S.R., and A.L. assisted with rearing of offspring and quantifying behavioural measurements, respectively. L.S. assisted with data collection for reproductive endpoints. S.B. provided the space and equipment for quantifying reproductive endpoints and assisted with statistical analyses. All authors provided editorial comments.

**CHAPTER 5: PROSTAGLANDINS PREVENT ACETAMINOPHEN INDUCED EMBRYO TOXICITY IN ZEBRAFISH (*Danio rerio*)**

Authors: Michal Galus, Akash Gugilla, Maria Jönsson and Joanna Y. Wilson.

Date Submitted: June 8, 2014

Journal: American Association of Pharmaceutical Scientists Journal

Comments: M.G. conducted this study and wrote the manuscript under the supervision of J.Y.W. A.G. assisted with the prostaglandin exposures. M.J. completed gene expression analyses. All authors provided editorial comments.

**CHAPTER 6: GENERAL SUMMARY AND CONCLUSIONS**

# CHAPTER 1

# GENERAL INTRODUCTION

## PHARMACEUTICALS IN THE ENVIRONMENT

Pharmaceuticals and personal care products (PPCPs) consist of diverse chemical compounds used in human, livestock and domestic animal health care and in the generation of human consumer products related to hygiene and beauty. Compounds with a similar mechanism of action or shared therapeutic/hygiene purpose, are categorized in the same pharmaceutical class. It is estimated that thousands of tons of prescription and non-prescription drugs are produced annually worldwide (Ellis, 2006; Halling-Sørensen et al., 1998; Morgan et al., 2011) and production is expected to rise with advancements in medical technology, increased spending on health care and aging demographics (Corcoran et al., 2010; Khetan and Collins, 2007). Typically, pharmaceutical compounds have high biological potency and produce their effects at low dose, raising concerns that environmental contamination may pose health risks for humans and aquatic species.

Pharmaceuticals were first identified as environmental contaminants of concern in 1999 (Daughton and Ternes, 1999) although their detection in the aquatic environment was as early 1965 when hormones, particularly estrogens, were shown to be present in effluent after conventional waste water treatment processes (Stumm-Zollinger and Fair, 1965). More recently, there have been significant advances in the detection of trace PPCPs in water and biological matrices and surveys have begun to well characterize the types and concentrations of PPCPs in wastewater treatment plant effluent (Brown et al., 2006; H. Jones et al., 2005; Karthikeyan and Meyer, 2006; Pedersen et al., 2005; Radjenović et al.,

2009; Stackelberg et al., 2004) surface water (Benotti and Brownawell, 2007; Kolpin et al., 2002; Metcalfe et al., 2003), ground water (Barnes et al., 2008; Watkinson et al., 2009), drinking water (Crane et al., 2006; Focazio et al., 2008), and biota (Huerta et al., 2012). The primary route of pharmaceutical entry into the environment is through human consumption; compounds are excreted as either a metabolite or parent compound and then make their way to municipal waste water treatment plants (WWTPs) (Lienert et al., 2007; Sedlak and Pinkston, 2011). A small percentage of PPCPs enter the environment through inappropriate disposal; involving either disposal of expired medication (Adams, 2009) or illegal dumping of waste (Bound and Voulvoulis, 2005; Glassmeyer et al., 2009; Ruhoy and Daughton, 2007; Vollmer, 2010). In agricultural areas, significant contributions of PPCPs to the environment are from agricultural runoff and animal wastes (Pedersen et al., 2005).

Generally, the classes of pharmaceuticals that are most frequently detected in the aquatic environment are correlated with those that are most heavily used (Benotti and Brownawell, 2007; Brun et al., 2006). Indeed, there are strong similarities in North America and Europe in the types and concentrations of pharmaceuticals that have been detected in receiving waters. Due to their high use, compounds from classes such as analgesics, antibiotics, antidepressants, anti-epileptics, beta blockers, lipid regulators, and some steroids are often detected within wastewater effluent and receiving waters (Andreozzi et al., 2003; Benotti and Brownawell, 2007; Benotti et al., 2008; Brun et al., 2006; Kolpin et al., 2002; Metcalfe et al., 2003; Sattelberger, 1999; Sedlak and Pinkston, 2011; Ternes, 1998; Tixier et al., 2003). Pharmaceuticals can by-pass treatment all together

through sewage overflow or they can persist in the environment as they are only marginally degraded in WWTP (Halling-Sørensen et al., 1998; Kolpin et al., 2002; Lau et al., 2002; Stackelberg et al., 2004). The reported concentrations of PPCPs range from high levels of µg $L^{-1}$ within wastewater effluent, as shown in Chapter 3 and (Metcalfe et al., 2010; Metcalfe et al., 2003; Trudeau et al., 2005) dropping too low µg $L^{-1}$ concentrations within receiving waters. In some cases, concentrations in receiving waters have been reported at low ng $L^{-1}$ (Domagalski et al., 2007; Halling-Sørensen et al., 1998; Kolpin et al., 2002). The large amount of data documenting pharmaceutical contamination of natural water systems provides evidence that these compounds are typically present at low levels in the aquatic environment, however the impacts of PPCPs in the environment on biota including fish and humans are less clear.

By design, pharmaceuticals induce biological responses in mammals at very low doses; suggesting their presence in lakes, rivers and streams is a potential concern for aquatic vertebrates within these ecosystems. Pharmaceuticals selectively target specific proteins in humans. Given that orthologs to human drug targets are present in fish and their genes have high sequence similarity to humans (Gunnarsson et al., 2008; Howe et al., 2013; Huggett et al., 2005), it is not unreasonable to expect that PPCPs in the environment could activate these same drug targets in fish. Fish are ecologically, culturally, and economically important species and numerous laboratory studies on the effects of PPCPs on aquatic systems have now been published.

## BIOLOGICAL IMPACTS OF PHARMACEUTICALS IN FISH SPECIES

*Analgesics and Anti-Inflammatories*

The most widely used analgesics and anti-inflammatories are non-prescription, non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are pain relievers that induce their biological response through inhibiting cyclooxygenase activity and reduction of endogenous prostaglandin production (Hawkey and Langman, 2003). In fish species, cyclooxygenase orthologs have been identified in many teleosts including rainbow trout (*Oncorhynchus mykiss*) (Zou et al., 1999), brook trout (*Salvelinus fontinalis;* Roberts et al., 2000) and zebrafish (*Danio rerio*) (Grosser et al., 2002). As in mammals, there are 2 *cox* genes in fish; *cox-1* which is responsible for basal prostaglandin production and *cox-2* which is inducible (Grosser et al., 2002; Ishikawa and Herschman, 2007). The mechanism behind NSAID toxicity in fish is not fully understood, however data presented in Chapter 5 strongly suggest that drugs of this class elicit their developmental toxicity in fish via the cyclooxygenase pathway.

NSAIDs are arguably one of the world's most widely used drug classes as they have been consistently detected in wastewater effluent and receiving waters at μg $L^{-1}$ concentrations all over the globe (Brun et al., 2006; Corcoran et al., 2010; Daughton and Ternes, 1999; Domagalski et al., 2007; Ferrari et al., 2004; H. Jones et al., 2005; Halling-Sørensen et al., 1998; Heberer, 2002; Huerta et al., 2012; Khetan and Collins, 2007; Kolpin et al., 2002; Metcalfe et al., 2003; Sattelberger, 1999; Ternes, 1998; Trudeau et al., 2005). Some of the most widely used NSAIDs include ibuprofen, acetaminophen, and naproxen in North America and diclofenac in Europe (Khetan and Collins, 2007). The low

concentrations of NSAIDs in the environment can be attributed to the fact that they are nearly 99% removed during conventional sewage treatment processes, as shown in Chapter 3 and (Kolpin et al., 2002; Metcalfe et al., 2003; Miao et al., 2002). However, their frequent use ensures that they are often detected in environmental samples.

Amongst fish, commonly reported effects of NSAID exposure include impacts on development. David and Pancharatna (2009) observed that zebrafish exposed at 10 -100 μg $L^{-1}$ of acetaminophen had reduced hatching and growth rates and altered swimming behaviour. At higher concentrations (50 – 100 μg $L^{-1}$) reductions in melanophore production were observed (David and Pancharatna, 2009). Similarly, Japanese medaka (*Oryzias latipes*) exposed at 95 mg $L^{-1}$ showed significant reduction in survival at 30 days post hatch (Kim et al., 2012). Exposure of fish to other NSAIDs like diclofenac and ibuprofen resulted in hatching delays and developmental impacts (Hallare et al., 2004; Han et al., 2010; Ji et al., 2013; Lee et al., 2011; Prášková et al., 2013; Van den Brandhof and Montforts, 2010), increased mortality (Stepanova et al., 2013) and reductions in feeding/feeding behaviour (Nassef et al., 2010b).

In adults, chronic NSAID exposure has been linked to histopathological alterations in the kidney and gill of fish (Hoeger et al., 2005; Mehinto et al., 2010; Peng et al., 2010; Schwaiger et al., 2004; Triebskorn et al., 2007). For example, rainbow trout exposed to 28 days of 1 μg $L^{-1}$ diclofenac (Schwaiger et al., 2004) showed regressive changes including structural alterations to cellular components within these organs. Furthermore, reproductive effects including alterations in the number of spawning events, clutch size and changes in

hormone concentrations have been linked with NSAID exposure (Flippin et al., 2007; Ji et al., 2013; Lister and Van Der Kraak, 2008).

Past research has shown consistent developmental effects and impacts on both kidney histology and reproduction in fish with NSAID exposure. However, many of these studies used concentrations that were well above 10 µg $L^{-1}$, the maximum reported concentration of PPCP in surface waters. Some studies, mostly using ibuprofen, have completed chronic exposures of NSAIDS with concentrations at or below 10 µg $L^{-1}$ (Flippin et al., 2007; Ji et al., 2013; Lee et al., 2011) but low concentration, chronic exposures are largely lacking for acetaminophen. In Chapter 2, developmental, histological and reproductive effects are reported for chronic exposures of zebrafish to acetaminophen at 0.5 and 10 µg $L^{-1}$. These novel findings are important for assessing the risk imposed on fish at environmentally relevant PPCP concentrations.

*Antibiotics*

Antibiotics are a group of pharmaceuticals frequently used in both human and veterinary medicine to inhibit the growth of microorganisms. The primary concern of antibiotics within receiving waters is the development of antibiotic resistant bacteria and the implications that may have on human health (Baquero et al., 2008; Boxall et al., 2012). In fact, antibiotics and antibiotic resistant bacteria have been documented in the environment but their highest concentrations are within WWTPs (Guardabassi and Dalsgaard, 2002; Hirsch et al., 1999; Kim and Aga, 2007; Kümmerer, 2003, 2009; Schlüter et al., 2007; Schwartz et al., 2003). There is the growing concern for proliferation of antibiotic resistant bacteria and transfer of resistant genes to nonresistant strains (Kruse and

Sørum, 1994; Poté et al., 2003). This could potentially lead to higher detection of antibiotic resistant bacteria within receiving waters, especially near sewage outflow points as discharge of effluent is the primary source of antibiotics and may be a source of antibiotic resistant bacteria (Kim and Aga, 2007).

Antibiotics incorporate many different mechanisms of action, however they always involve interfering with cellular components required for bacterial reproduction (Sanderson et al., 2004). Unlike many pharmaceuticals, antibiotics are specifically designed to target only the bacterial components, thus we do not expect their effect to follow a typical dose response relationship. A few studies have examined the impacts of antibiotics in fish, particularly sulphonamides. These compounds were shown to be acutely toxic to fish when exposed at concentrations well above 100 mg $L^{-1}$; values that have never been found in the North American or European environment (Kim et al., 2007; Kubota et al., 1970; Snieszko and Friddle, 1951) but have been found in India (Fick et al., 2009; Larsson et al., 2007). More recently, the antibiotic triclosan has shown to be acutely toxic to fish with reported impacts on development and hatching (Ishibashi et al., 2004; Nassef et al., 2010b; Oliveira et al., 2009), sperm production (Raut and Angus, 2010) and social behaviour (Fritsch et al., 2013). It is more likely that antibiotics within the environment may have an indirect effect on fish populations by potentially impacting food sources at lower tropic levels, rather than directly effecting fish physiology (Costanzo et al., 2005; Kümmerer, 2003).

*Anti-Depressants*

The most common type of anti-depressants are the selective serotonin reuptake inhibitors (SSRIs), which include fluoxetine, sertraline or citalopram. They are generally

detected at concentrations ranging from ng $L^{-1}$ to μg $L^{-1}$ due to their poor removal rate (~30%) from WWTPs (Andreozzi et al., 2003; Kolpin et al., 2002; Metcalfe et al., 2003). These pharmaceuticals elicit their therapeutic action by altering the reabsorption of neurotransmitters, specifically serotonin, at the synaptic cleft resulting in elevated intracellular concentrations and altered mood (Fent et al., 2006). More recently, a new class of anti-depressants, serotonin and norepinephrine reuptake inhibitors (SNRIs) have emerged as a popular alternative to SSRIs. These compounds function in a similar mechanism to SSRIs, however they prevent the reabsorption of both serotonin and norepinephrine. Much like serotonin, norepinephrine is a key neurotransmitter involved in mood regulation; thus the additional targeting that SNRIs provide allows for a more potent pharmacological response (Sussman, 2003).

Serotonin is an important neurotransmitter in fish as it has been linked to various physiological processes including regulating feeding and breeding behaviour (Mennigen et al., 2010a; Mennigen et al., 2008), appetite (Mennigen et al., 2010b) and establishing social hierarchies (Mennigen et al., 2011; Metcalfe et al., 2010). Much of the work on SSRIs has been carried out with fluoxetine and there are many studies that elegantly demonstrate its role as a neuroendocrine disruptor (as reviewed in Trudeau et al., 2011; Trudeau et al., 2005; Waye and Trudeau, 2011).

Microgram concentrations of fluoxetine (0.1 – 5 μg $L^{-1}$) caused severe developmental abnormalities in Japanese medaka including curvature of the spine, reduced eye size or even lack of fins (Foran et al., 2004). Many studies with fluoxetine have documented alterations in behaviour across many fish species ranging from reduced

locomotor activity (Airhart et al., 2007), decreased responsiveness to stimulation (Foran et al., 2004) and reduced predator avoidance (Painter et al., 2009; Weinberger II and Klaper, 2014). A decrease in feeding and feeding behaviour was commonly reported as an impact of fluoxetine exposure (10 – 100 μg $L^{-1}$) (Gaworecki and Klaine, 2008; Mennigen et al., 2009; Mennigen et al., 2010b; Stanley et al., 2007; Weinberger II and Klaper, 2014).

Exposure to fluoxetine has been linked to impacts in reproduction. Bluehead wrasse (*Thalassoma bifasciatum*) injected at 6 μg $g^{-1}$ body weight showed increased aggression during mating (Perreault et al., 2003), an effect which was observed in fathead minnows exposed at 100 μg $L^{-1}$ (Weinberger II and Klaper, 2014). Zebrafish exposed at 32 μg $L^{-1}$ showed reduced fecundity (Lister et al., 2009) and male goldfish had decreased milt production (Mennigen et al., 2010a) which could explained by changes in hormone concentrations (Foran et al., 2004; Mennigen et al., 2008). Given that all SSRIs and SNRIs function under a common mechanism, we may expect that the endpoints would be similar regardless of compound choice. SNRIs have not been tested for impacts on the neuroendocrine or reproductive systems.

In Chapter 2, reproductive and hormonal impacts of chronic exposure of zebrafish to venlafaxine, a SNRI commonly found in the aquatic environment, was examined. These novel findings suggest that SNRIs can elicit similar physiological impacts compared to those documented in SSRIs and future studies would benefit from a more detailed analysis of this class of anti-depressants.

*Anti-Epileptics and Mood Stabilizers*

Anti-epileptics are a class of pharmaceuticals prescribed primarily to mitigate symptoms of epilepsy such as the frequency of seizures or degree of tremors. Although the exact mechanisms of action behind anti-epileptics are not well known, they elicit their pharmacological effects by altering the degree of neuronal excitation and permeability of various intracellular ions (Kwan et al., 2001). Pharmaceuticals in this class target voltage gated $Na^{+}$, $K^{+}$ or $Ca^{2+}$ channels and modulation of the availability and permeability of the neurotransmitter γ-aminobutyric acid (GABA) (Meldrum, 1995, 1996). Carbamazepine is one of the most persistent PPCPs in the environment with an average half-life of 82 days and has been be detected at distances nearly 2 km away from wastewater effluent outflow points (Hoque et al., 2014; Lam et al., 2004). Given the poor removal rates during conventional waste-water treatment and high stability of anti-epileptics within various aquatic environments, it is surprising that there are only a few studies examining their impacts on fish; and only one that used environmentally relevant concentrations (Triebskorn et al., 2007).

Carbamazepine injected into Japanese medaka embryos at 12 ng $egg^{-1}$ resulted in delayed development and increase mortality throughout embryogenesis (Nassef et al., 2010a). At 6.5 mg $L^{-1}$ reductions in swimming speed and feeding rate were also observed in the same species (Nassef et al., 2010). Furthermore, common carp (*Cyprinus carpio*) exposed at 20 -100 μg $L^{-1}$ had altered gill and liver structure (Triebskorn et al., 2007) whereas zebrafish exposed to concentrations well above those detected in the environment (>30 mg $L^{-1}$) showed reduced growth rate (Van den Brandhof and Montforts, 2010).

Chapters 2 and 4 expanded the knowledge base on the physiological impacts of anti-epileptic exposure on fish species. I have examined the effects of carbamazepine on reproduction, organ histology and plasma steroid concentrations in adults (Chapter 2). Developmental effects of carbamazepine were assessed with direct exposure to zebrafish embryos (Chapter 2); the effects of parental exposure to carbamazepine on offspring were examined during early embryogenesis (Chapter 2) and at sexual maturity (Chapter 4). In all cases, concentrations were at maximum 10 ug $L^{-1}$ and showed that environmentally relevant concentrations can disrupt multiple physiological systems.

*Beta Blockers*

Beta blockers or β-adrenergic receptor antagonists are important in the treatment and prevention of cardiovascular diseases. They are often prescribed to treat hypertension, cardiac arrhythmia or to regulate cardiac workload (Owen et al., 2007). β-Blockers elicit their effects in humans by inhibiting the β1, β2 and β3 receptors, resulting in reduced heart rate and lowered blood pressure (Weber, 2005). In spite of the their high usage, β-blockers have high removal rates from WWTP and thus, are generally detected at ng $L^{-1}$ concentrations within receiving waters (Andreozzi et al., 2003; Ferrari et al., 2004; Huggett et al., 2005; Khetan and Collins, 2007; Ternes, 1998; Zuccato et al., 2000).

The β-adrenergic receptors are well conserved across vertebrate taxa and have been identified in many fish tissues (Owen et al., 2007). Much like in mammals these receptors can be found in organs outside of those directly involved with cardiac function (Owen et al., 2007). The identification of these receptors in fish gills, liver brain and muscle implies that β-blockers have the potency to impact many physiological processes in fish, in

particular those involved in cardiac function, metabolism, growth and reproduction (Lortie and Moon, 2003; Massarsky et al., 2011; Nickerson et al., 2001; Nickerson et al., 2003; Owen et al., 2007; Zikopoulos and Dermon, 2005) .

Rainbow trout injected with 2 mg $kg^{-1}$ of propranonol had decreased heart rate, yet aqueous exposure (70 μg $L^{-1}$) had no impact (Larsson et al., 2006). Propranonol has been shown to interfere with glycogenolysis (Mommsen et al., 1988; Owen et al., 2009) and 1479 μg $L^{-1}$ exposure reduced hepatic glucose production by 92% (McKinlkey and Hazel, 1993). Reduction in intracellular glucose stores could be responsible for the reduced growth rates observed in 14 day, 0.5 μg $L^{-1}$ exposed Japanese medaka (Huggett et al., 2002) and 40 day, 10 mg $L^{-1}$ exposed rainbow trout (Owen et al., 2009).

Adult fathead minnows (*Pimephales promelas*) exposed for 21 days to 1 mg $L^{-1}$ propranolol showed reduced fecundity and hatching delays and increased gonado-somatic index of both sexes; impacts which were not observed in concentrations below 10 μg $L^{-1}$ (Giltrow et al., 2009). Although there have been several studies that examine β-blocker toxicity, many used concentrations that were not environmentally relevant (Ferrari et al., 2004; Fraysse et al., 2006; Larsson et al., 2006; Winter et al., 2008), yet these studies suggest that effects may not be likely at environmentally relevant doses because no observable effects concentrations were well above environmental concentrations.

*Lipid Regulators*

Fibrates are a class of pharmaceuticals which are clinically prescribed to lower plasma triglycerides and total cholesterol (Spencer and Barradell, 1996). All fibrates are

peroxisome proliferators and although their exact mechanism of action is unclear it is believed that they elicit their effects by binding to the nuclear transcription factor peroxisome proliferator-activated receptor-alpha (PPARα) and promoting hepatic uptake and metabolism of free fatty acids (Martin et al., 1997). Fibrate lipid regulators are a heavily used class of pharmaceuticals, which explains why they are frequently detected in the environment at $\mu g\ L^{-1}$ concentrations (Miao et al., 2002; Sumpter, 2007; Trudeau et al., 2005). However, these compounds are only ~40% removed during conventional sewage treatment processes (Chapter 3, Isidori et al., 2007; Khetan and Collins, 2007) .

Activation of the PPARα pathway results in the generation of reactive oxygen species and oxidative stress on various intracellular components and organs, particularly the liver. Clofibrate, a commonly prescribed fibrate, has been associated with oxidative stress in hepatic and gill tissue in eastern mosquito fish (*Gambusia holbrooki*) (Nunes et al., 2008). This increase in reactive oxygen species could also explain the histopathology in the liver, kidney and gill observed in rainbow trout exposed at 5 - 100 $\mu g\ L^{-1}$ (Triebskorn et al., 2007). Zebrafish exposed to clofibrate 0.1 – 1 mg $L^{-1}$ showed an increase in time to hatch as well as reduced consumption of the yolk during embryogenesis (Raldúa et al., 2008), an endpoint documented with exposure to gemfibrozil but at concentrations of 5 mg $L^{-1}$ (Raldúa et al., 2008) These impacts where more severe with clofibrate exposure and the authors concluded that beyond the effects with the PPARα system, clofibrate may be inhibiting the yolk syncytial layer; contributing to the low yolk consumption observed (Raldúa et al., 2008).

Reproductive impacts have been documented with clofibrate and gemfibrozil exposure. Chronic exposure to fathead minnows at of 0.01 – 1 mg $L^{-1}$ clofibrate resulted in impaired spermatogenesis, reduced sperm count and lower plasma androgen concentrations (Runnalls et al., 2007). Fathead minnows exposed to 1.5 mg $L^{-1}$ of gemfibrozil had reduced fecundity (Skolness et al., 2012). The impacts on reproduction could stem from hormonal imbalance as fibrate exposure has shown to reduce plasma testosterone concentrations in many fish species (Mimeault et al., 2005; Prindiville et al., 2011; Skolness et al., 2012). For example, goldfish (*Carassius auratus)* exposed to gemfibrozil at 1500 μg $L^{-1}$ for 96h had up to 50% reductions of plasma testosterone. The authors believed this occurred through a reduction of the steroid acute regulatory protein, which is responsible for the transport of cholesterol during steroidogenesis (Mimeault et al., 2005).

A majority of the work characterizing fibrate exposure in fish has been done at concentrations outside of what is found in the environment. Chapters 2 and 4, fill this gap and document that chronic exposure to gemfibrozil at 0.5 and 10 μg $L^{-1}$ leads to atretic oocytes and altered ovarian histology, coupled with decreased reproduction, in female zebrafish. No studies have examined parental impacts of fibrate exposure on offspring and in Chapter 4, I describe how parental exposure to 10 μg $L^{-1}$ gemfibrozil reduced fecundity, altered male breeding behaviours and altered sperm (morphology and velocity) in offspring.

## ZEBRAFISH AS A MODEL ORGANISIM

The zebrafish (*Danio rerio*), a small tropical fish species endemic to the Ganges River, has traditionally been used as a model organism in developmental and molecular

biology, but more recently it has become increasingly useful for environmental and comparative toxicology studies (Langheinrich, 2003; McGrath and Li, 2008; Scholz et al., 2008). Many characteristics make the zebrafish an appealing model organism, such as their ease of manipulation (Scholz et al., 2008), frequent spawning (Briggs, 2002; Kimmel et al., 1995; Wixon, 2000), external fertilization (Briggs, 2002; Wixon, 2000), large number of offspring (Briggs, 2002; Kimmel et al., 1995; Wixon, 2000), transparent embryos (Kimmel et al., 1995; McGrath and Li, 2008), short generation time (Briggs, 2002; Kimmel et al., 1995) and fully sequenced genome (Woods et al., 2000). For the objectives of this thesis, zebrafish were an appealing fish model due to their reproductive and developmental characteristics.

In the laboratory, zebrafish can be housed at relatively high densities, in small aquaria reducing the husbandry time and costs. Females are capable of reproducing all year round and usually produce 20 – 250 embryos per mating event. This was a significant advantage for this thesis as reproduction was an endpoint of interests and with female fish producing large numbers of embryos, impacts on fecundity were easily identifiable. Fertilization of the embryos occurs externally and embryogenesis is rapid with the main body plan developed by 24 hpf and all organs fully developed by 120 – 136 hpf (Kimmel et al., 1995), allowing for chronic developmental exposures within a short time frame for a vertebrate organism. The clear chorion makes direct, live observations of the development of the fish possible, and allowed for continuous observation of single embryos throughout development. The small size of zebrafish embryos is beneficial as they can be easily manipulated and develop normally in microplates allowing for easy screening of large

quantities of animals. Once the larval fish have broken free of the chorion and are free swimming (~96 hpf), they can reach sexual maturity in 4 – 5 months, allowing for a more reasonable time to assess effects on offspring through to sexual maturity.

Zebrafish are one of few fish species who have their morphological and physiological characteristics carefully documented from the onset of fertilization, through sexual maturity in adults. Coupled with the transparency of their embryos, this makes zebrafish particularly useful for assessing any developmental consequences from exposure to endocrine disrupting chemicals. Furthermore, the fully sequenced genome allows for robust gene expression analyses allowing a comprehensive understanding of target genes or biochemical pathways possibly effected.

Chemical exposure may result in cellular or structural tissue damage such as necrosis, apoptosis or other histopathological alterations. Much like the developmental atlas, there are resources for zebrafish (www.zfin.org, zfatlas.psu.edu) that outline normal tissue histology, providing important resources to identify tissue specific changes. The possibility of chemicals altering behaviour has been documented in zebrafish (Darrow and Harris, 2004). Fortunately, social and reproductive behaviours have been well characterized for this species (Darrow and Harris, 2004). For example, courtship behaviour amongst zebrafish is almost exclusively a male activity (Darrow and Harris, 2004). The male will use a series of easily identifiable displays to initiate spawning with a female. If these behaviours are absent from the courtship display, the likelihood of spawning is reduced (Darrow and Harris, 2004). Therefore, when assessing if chemicals are impacting

behaviour, the knowledge of what is normal and abnormal contributes to the ease of assessing toxicity of a given compound.

The zebrafish represents a model species with a wide range of possible applications. For this thesis, the zebrafish was an ideal model organism as it provided a means to assess the toxicity of specific PPCPs and PPCP mixtures to development, reproduction, steroid hormone concentrations, organ histology, and courtship behavior.

## THESIS GOALS

The overall objective of this thesis was to examine the physiological changes in zebrafish, both embryonic and adult, exposed to environmentally relevant concentrations of single pharmaceuticals and pharmaceutical mixtures including wastewater effluent. These findings provide insight into the toxicity associated with PPCP exposure at environmentally relevant concentrations. Through understanding the impacts low concentrations of pharmaceuticals have on biological processes, we will be better able to predict the toxicity of these compounds and extrapolate potential effects to native fish species in environmental systems.

The pharmaceuticals acetaminophen, carbamazepine, gemfibrozil and venlafaxine were used as test compounds for this study as they are representatives of commonly detected pharmaceutical classes in environmental waters. As pharmaceutical exposure in the environment is never to only one compound, we conducted one study involving wastewater effluent and pharmaceutical mixtures to better understand real world exposure scenarios. Humans can experience toxicity from drug-drug interactions when exposed to

multiple pharmaceuticals. Pharmaceutical interactions are commonly mediated by alterations in pharmacokinetics; altering the toxicity of one or more compounds. In some cases, individually safe, therapeutic drugs become toxic when combined, causing adverse side effects (Kang et al., 2014). As fish in the wild are always exposed to mixtures of these compounds, it was important to understand whether the toxicity identified with single compound exposure were relevant for mixtures.

The four PPCP tested represent different pharmaceutical classes found in the environment at environmentally relevant concentrations, albeit the high concentration was near the highest reported concentrations detected. Since complex PPCP mixtures are found in the environment, many pharmaceuticals are present with similar modes of action and thus the high concentration was representative of the concentrations for all PPCPs within a single drug class. Thus, the concentrations are not meant to be an exact match to what is found at one wastewater treatment plant, or field site, but to represent concentrations that are reasonable and environmentally relevant.

The chronic toxicity of PPCPs was assessed using the zebrafish model, an important model fish species with clearly documented development, allowing for easy identification of adverse embryonic effects from chemical exposure. A continuous spawning species, they are well accepted as a model species for reproductive toxicity. Their short generation time allowed for an assessment of the effects of parental exposure of offspring. Thus, the specific goals of this thesis were to:

1. Examine the impacts of chronic pharmaceutical exposure in embryonic and adult zebrafish with exposure to environmentally relevant concentrations of acetaminophen, carbamazepine, gemfibrozil and venlafaxine.

2. Examine the impacts of chronic exposure to pharmaceutical mixtures in embryonic and adult zebrafish with exposure at environmentally relevant concentrations.

3. Examine the impacts of parental exposure to pharmaceuticals on offspring reproduction. Zebrafish offspring were raised from parents chronically exposed to environmentally relevant concentrations of carbamazepine and gemfibrozil.

4. Characterize developmental effects of acetaminophen on zebrafish embryos and determine if acetaminophen elicits its developmental toxicity in fish through the cyclooxygenase pathway.

**CHAPTER SUMMARY**

Although zebrafish have been used in many studies to demonstrate adverse effects from pharmaceutical exposure, a majority of studies tested compounds under acute conditions and with concentrations well above environmental levels. This work significantly contributes to the field of aquatic toxicology as it demonstrates that chronic exposure to low levels of pharmaceuticals can impact multiple life stages and organ system in fish species.

Chapter 2 examined the chronic impact of aqueous, pharmaceutical exposure on the model vertebrate *Danio rerio*. Environmentally relevant concentrations of pharmaceuticals were capable of reducing the fecundity in spawning adults and impacting embryogenesis in offspring. All compounds significantly reduced reproductive output, kidney histology and embryonic mortality. Histological examination of reproductive organs and hormone

analyses were conducted in an effort to understand the reproductive impacts observed from PPCP exposure.

Chapter 3 examined the chronic impact of aqueous pharmaceutical mixtures in zebrafish, exposures which are most relevant to fish under real world exposure scenarios. Endpoints were identical to those applied in Chapter 2, in animals treated with a simple pharmaceutical mixture and diluted wastewater effluent. The pharmaceutical mixture was comprised of the four individual PPCPs studied in Chapter 2. Wastewater effluent exposures were a more complicated mixture as there were PPCPs, ammonia and other contaminants present. These studies help further validate that single dose exposures can identify relevant endpoints for studying PPCP mixtures and wastewater effluent in adult and embryonic fish.

Chapter 4 focused on examining the effects of chronic parental pharmaceutical exposure on zebrafish offspring. Embryos from chronically exposed parents were reared to sexual maturity to assess if reproduction was impacted from parental exposure alone. Furthermore, sex specific effects were determined to identify if male or female offspring were more impacted by parental exposure. Whether these impacts are due to impacts on gametogenesis or from maternal transfer of contaminants to the egg was not identified.

Chapter 5 focused on the toxicity of acetaminophen in embryonic zebrafish. The goal was to determine if acetaminophen induced mortality and abnormality in embryonic zebrafish was mediated via the cyclooxygenase pathway. This study showed that cyclooxygenase genes were expressed and functional during gastrulation and segmentation.

Acetaminophen exposed animals were supplemented with exogenous prostaglandin E2, a major product of the cyclooxygenase pathway, to determine if acetaminophen exposure was decreasing protaglandin production and eliciting its toxic response in zebrafish under the same mechanism of action for therapeutic effects in mammals.

Chapter 6 summarizes the key findings from the experimental chapters (Chapter 2-5) in this thesis and discusses how these data identify the risks that aquatic species face from chronic, low concentration PPCP exposure. Chapter 6 provides perspectives on the findings and discusses potential routes for future research.

**REFERENCES**

Adams, M., 2009. India's Waterways A Toxic Stew of Pharmaceutical Chemicals Dumped from Big Pharma Factories. Natural News.

Airhart, M.J., Lee, D.H., Wilson, T.D., Miller, B.E., Miller, M.N., Skalko, R.G., 2007. Movement disorders and neurochemical changes in zebrafish larvae after bath exposure to fluoxetine (PROZAC). Nerotoxicol Teratol 29, 652-664.

Andreozzi, R., Raffaele, M., Nicklas, P., 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere 50, 1319-1330.

Baquero, F., Martínez, J.-L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water environments. Curr Opin Biotech 19, 260-265.

Barnes, K.K., Kolpin, D.W., Furlong, E.T., Zaugg, S.D., Meyer, M.T., Barber, L.B., 2008. A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States—I) Groundwater. Sci Total Environ 402, 192-200.

Benotti, M.J., Brownawell, B.J., 2007. Distributions of pharmaceuticals in an urban estuary during both dry- and wet-weather conditions. Environ Sci Technol 41, 5795-5802.

Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder, S.A., 2008. Pharmaceuticals and endocrine disrupting compounds in US drinking water. Environ Sci Technol 43, 597-603.

Bound, J.P., Voulvoulis, N., 2005. Household disposal of pharmaceuticals as a pathway for aquatic contamination in the United Kingdom. Environ Health Persp, 1705-1711.

Boxall, A.B., Rudd, M.A., Brooks, B.W., Caldwell, D.J., Choi, K., Hickmann, S., Innes, E., Ostapyk, K., Staveley, J.P., Verslycke, T., 2012. Pharmaceuticals and personal care products in the environment: what are the big questions? Environ Health Persp 120, 1221.

Brown, K.D., Kulis, J., Thomson, B., Chapman, T.H., Mawhinney, D.B., 2006. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. Sci Total Environ 366, 772-783.

Brun, G.L., Bernier, M., Losier, R., Doe, K., Jackman, P., Lee, H.B., 2006. Pharmaceutically active compounds in atlantic canadian sewage treatment plant effluents and receiving waters, and potential for environmental effects as measured by acute and chronic aquatic toxicity. Environ Toxicol Chem 25, 2163-2176.

Corcoran, J., Winter, M.J., Tyler, C.R., 2010. Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. Crit Rev Toxicol 40, 287-304.

Costanzo, S.D., Murby, J., Bates, J., 2005. Ecosystem response to antibiotics entering the aquatic environment. Mar Pollut Bull 51, 218-223.

Crane, M., Watts, C., Boucard, T., 2006. Chronic aquatic environmental risks from exposure to human pharmaceuticals. Sci Total Envrion 367, 23-41.

Darrow, K.O., Harris, W.A., 2004. Characterization and development of courtship in zebrafish, *Danio rerio*. Zebrafish 1, 40-45.

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ Health Persp 107, 907.

David, A., Pancharatna, K., 2009. Effects of acetaminophen (paracetamol) in the embryonic development of zebrafish, *Danio rerio*. J Appl Toxicol 29, 597-602.

Domagalski, J., Belitz, K., Furlong, E., 2007. Occurrence of pharmaceutical and other organic compounds in ground water in ten regions of California, USA, AGU Spring Meeting Abstracts, p. 03.

Ellis, J.B., 2006. Pharmaceutical and personal care products (PPCPs) in urban receiving waters. Environ Poll 144, 184-189.

Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat Toxicol 76, 122-159.

Ferrari, B., Mons, R., Vollat, B., Fraysse, B., Paxēaus, N., Giudice, R.L., Pollio, A., Garric, J., 2004. Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? Environ Toxicol Chem 23, 1344-1354.

Fick, J., Söderström, H., Lindberg, R.H., Phan, C., Tysklind, M., Larsson, D., 2009. Contamination of surface, ground, and drinking water from pharmaceutical production. Environ Toxicol Chem 28, 2522-2527.

Flippin, J.L., Huggett, D., Foran, C.M., 2007. Changes in the timing of reproduction following chronic exposure to ibuprofen in Japanese medaka, *Oryzias latipes*. Aquat Toxicol 81, 73-78.

Focazio, M.J., Kolpin, D.W., Barnes, K.K., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Barber, L.B., Thurman, M.E., 2008. A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States—II) Untreated drinking water sources. Sci Total Environ 402, 201-216.

Foran, C.M., Weston, J., Slattery, M., Brooks, B.W., Huggett, D.B., 2004. Reproductive assessment of Japanese medaka (*Oryzias latipes*) following a four-week fluoxetine (SSRI) exposure. Arch Environ Con Tox 46, 511-517.

Fraysse, B., Mons, R., Garric, J., 2006. Development of a zebrafish 4-day embryo-larval bioassay to assess toxicity of chemicals. Ecotox Environ Safe 63, 253-267.

Fritsch, E.B., Connon, R.E., Werner, I., Davies, R.E., Beggel, S., Feng, W., Pessah, I.N., 2013. Triclosan impairs swimming behavior and alters expression of excitation-contraction coupling proteins in fathead minnow (*Pimephales promelas*). Environ Sci Technol 47, 2008-2017.

Gaworecki, K.M., Klaine, S.J., 2008. Behavioral and biochemical responses of hybrid striped bass during and after fluoxetine exposure. Aquat Toxicol 88, 207-213.

Giltrow, E., Eccles, P.D., Winter, M.J., McCormack, P.J., Rand-Weaver, M., Hutchinson, T.H., Sumpter, J.P., 2009. Chronic effects assessment and plasma concentrations of the β-blocker propranolol in fathead minnows (*Pimephales promelas*). Aquat Toxicol 95, 195-202.

Glassmeyer, S.T., Hinchey, E.K., Boehme, S.E., Daughton, C.G., Ruhoy, I.S., Conerly, O., Daniels, R.L., Lauer, L., McCarthy, M., Nettesheim, T.G., 2009. Disposal practices for unwanted residential medications in the United States. Environ Int 35, 566-572.

Grosser, T., Yusuff, S., Cheskis, E., Pack, M.A., FitzGerald, G.A., 2002. Developmental expression of functional cyclooxygenases in zebrafish. PNAS 99, 8418-8423.

Guardabassi, L., Dalsgaard, A., 2002. Occurrence and fate of antibiotic resistant bacteria in sewage.
Danish EPA Envrionmental Project Report 722.

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.J., 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ Sci Technol 42, 5807-5813.

H. Jones, O., Voulvoulis, N., Lester, J., 2005. Human pharmaceuticals in wastewater treatment processes. Crit Rev Environ Sci Technol 35, 401-427.

Hallare, A., Köhler, H.-R., Triebskorn, R., 2004. Developmental toxicity and stress protein responses in zebrafish embryos after exposure to *diclofenac* and its solvent, DMSO. Chemosphere 56, 659-666.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P., Ingerslev, F., Holten Lützhøft, H., Jørgensen, S., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment-a review. Chemosphere 36, 357-393.

Han, S., Choi, K., Kim, J., Ji, K., Kim, S., Ahn, B., Yun, J., Choi, K., Khim, J.S., Zhang, X., 2010. Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*. Aquat Toxicol 98, 256-264.

Hawkey, C., Langman, M., 2003. Non-steroidal anti-inflammatory drugs: overall risks and management. Complementary roles for COX-2 inhibitors and proton pump inhibitors. Gut 52, 600-608.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol Lett 131, 5-17.

Hirsch, R., Ternes, T., Haberer, K., Kratz, K.-L., 1999. Occurrence of antibiotics in the aquatic environment. Sci Total Environ 225, 109-118.

Hoeger, B., Köllner, B., Dietrich, D.R., Hitzfeld, B., 2005. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta fario*). Aquat Toxicol 75, 53-64.

Hoque, M.E., Cloutier, F., Arcieri, C., McInnes, M., Sultana, T., Murray, C., Vanrolleghem, P.A., Metcalfe, C.D., 2014. Removal of selected pharmaceuticals, personal care products and artificial sweetener in an aerated sewage lagoon. Sci Total Environ.

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature.

Huerta, B., Rodríguez-Mozaz, S., Barceló, D., 2012. Pharmaceuticals in biota in the aquatic environment: analytical methods and environmental implications. Anal Bioanal Chem 404, 2611-2624.

Huggett, D., Benson, W., Chipman, K., Cook, J., Gray, L., Kinter, L., Meyerhoff, R., Trudeau, V., 2005. Role of mammalian data in determining pharmaceutical responses in aquatic species. SETAC Press, Pensacola, FL.

Huggett, D., Brooks, B., Peterson, B., Foran, C., Schlenk, D., 2002. Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-blockers) on aquatic organisms. Arch Environ Con Tox 43, 229-235.

Ishibashi, H., Matsumura, N., Hirano, M., Matsuoka, M., Shiratsuchi, H., Ishibashi, Y., Takao, Y., Arizono, K., 2004. Effects of triclosan on the early life stages and reproduction of medaka *Oryzias latipes* and induction of hepatic vitellogenin. Aquat Toxicol 67, 167-179.

Ishikawa, T.-o., Herschman, H.R., 2007. Two inducible, functional cyclooxygenase-2 genes are present in the rainbow trout genome. J Cell Biochem 102, 1486-1492.

Isidori, M., Nardelli, A., Pascarella, L., Rubino, M., Parrella, A., 2007. Toxic and genotoxic impact of fibrates and their photoproducts on non-target organisms. Envrion Int 33, 635-641.

Ji, K., Liu, X., Lee, S., Kang, S., Kho, Y., Giesy, J.P., Choi, K., 2013. Effects of non-steroidal anti-inflammatory drugs on hormones and genes of the hypothalamic-pituitary-gonad axis, and reproduction of zebrafish. J Hazard Mater 254, 242-251.

Kang, M.H., Villablanca, J.G., Bender, J.L.G., Matthay, K.K., Groshen, S., Sposto, R., Czarnecki, S., Ames, M.M., Reynolds, C.P., Marachelian, A., 2014. Probable fatal drug interaction between intravenous fenretinide, ceftriaxone, and acetaminophen: a case report from a New Approaches to Neuroblastoma (NANT) Phase I study. BMC research notes 7, 256.

Karthikeyan, K., Meyer, M.T., 2006. Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. Sci Total Environ 361, 196-207.

Khetan, S.K., Collins, T.J., 2007. Human pharmaceuticals in the aquatic environment: a challenge to green chemistry. Chem Rev 107, 2319-2364.

Kim, P., Park, Y., Ji, K., Seo, J., Lee, S., Choi, K., Kho, Y., Park, J., Choi, K., 2012. Effect of chronic exposure to acetaminophen and lincomycin on Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*, and potential mechanisms of endocrine disruption. Chemosphere 89, 10-18.

Kim, S., Aga, D.S., 2007. Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. J Toxicol Environ Health B 10, 559-573.

Kim, Y., Choi, K., Jung, J., Park, S., Kim, P.-G., Park, J., 2007. Aquatic toxicity of acetaminophen, carbamazepine, cimetidine, diltiazem and six major sulfonamides, and their potential ecological risks in Korea. Environ Int 33, 370-375.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater

contaminants in US streams, 1999-2000: A national reconnaissance. Environ Sci Technol 36, 1202-1211.
Kruse, H., Sørum, H., 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Appl Environ Microb 60, 4015-4021.

Kubota, S., Kojima, S., Ishida, A., 1970. A side effect of sulfonamides in fish. Fish Pathology 4, 98-102.

Kümmerer, K., 2003. Significance of antibiotics in the environment. J AntiMicrob Chemoth 52, 5-7.

Kümmerer, K., 2009. Antibiotics in the aquatic environment–a review–part I. Chemosphere 75, 417-434.

Kwan, P., Sills, G.J., Brodie, M.J., 2001. The mechanisms of action of commonly used antiepileptic drugs. Pharmacol Therapeut 90, 21-34.

Lam, M.W., Young, C.J., Brain, R.A., Johnson, D.J., Hanson, M.A., Wilson, C.J., Richards, S.M., Solomon, K.R., Mabury, S.A., 2004. Aquatic persistence of eight pharmaceuticals in a microcosm study. Environ Toxicol Chem 23, 1431-1440.

Larsson, D., de Pedro, C., Paxeus, N., 2007. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. J Hazard Mater 148, 751-755.

Larsson, D., Fredriksson, S., Sandblom, E., Paxeus, N., Axelsson, M., 2006. Is heart rate in fish a sensitive indicator to evaluate acute effects of β-blockers in surface water? Environ Toxicol Phar 22, 338-340.

Lau, J., Butler, D., Schütze, M., 2002. Is combined sewer overflow spill frequency/volume a good indicator of receiving water quality impact? Urban water 4, 181-189.

Lee, J., Ji, K., Lim Kho, Y., Kim, P., Choi, K., 2011. Chronic exposure to diclofenac on two freshwater cladocerans and Japanese medaka. Ecotox Environ Safe 74, 1216-1225.

Lienert, J., Güdel, K., Escher, B.I., 2007. Screening method for ecotoxicological hazard assessment of 42 pharmaceuticals considering human metabolism and excretory routes. Environ Sci Technol 41, 4471-4478.

Lister, A., Regan, C., Van Zwol, J., Van Der Kraak, G., 2009. Inhibition of egg production in zebrafish by fluoxetine and municipal effluents: A mechanistic evaluation. Aquat Toxicol 95, 320-329.

Lister, A., Van Der Kraak, G., 2008. An investigation into the role of prostaglandins in zebrafish oocyte maturation and ovulation. Gen Comp Endocr 159, 46-57.

Lortie, M.B., Moon, T.W., 2003. The rainbow trout skeletal muscle β-adrenergic system: characterization and signaling. Am J Phsyiol - Reg I 284, R689-R697.

Martin, G., Schoonjans, K., Lefebvre, A.-M., Staels, B., Auwerx, J., 1997. Coordinate Regulation of the Expression of the Fatty Acid Transport Protein and Acyl-CoA Synthetase Genes by PPARα and PPARγ Activators. J Biol Chem 272, 28210-28217.

Massarsky, A., Trudeau, V.L., Moon, T.W., 2011. β-blockers as endocrine disruptors: the potential effects of human β-blockers on aquatic organisms. J Exp Zool A 315, 251-265.

McKinlkey, S.J., Hazel, J.R., 1993. Epinephrine stimulation of glucose release from perfused trout liver: effects of assay and acclimation temperature. J Exp Biol 177, 51-62.

Mehinto, A.C., Hill, E.M., Tyler, C.R., 2010. Uptake and biological effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*). Environ Sci Technol 44, 2176-2182.

Meldrum, B.S., 1995. Neurotransmission in epilepsy. Epilepsia 36, 30-35.

Meldrum, B.S., 1996. Update on the mechanism of action of antiepileptic drugs. Epilepsia 37, S4-S11.

Mennigen, J.A., Harris, E.A., Chang, J.P., Moon, T.W., Trudeau, V.L., 2009. Fluoxetine affects weight gain and expression of feeding peptides in the female goldfish brain. Regul Peptides 155, 99-104.

Mennigen, J.A., Lado, W.E., Zamora, J.M., Duarte-Guterman, P., Langlois, V.S., Metcalfe, C.D., Chang, J.P., Moon, T.W., Trudeau, V.L., 2010a. Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish,*Carassius auratus*. Aquat Toxicol 100, 354-364.

Mennigen, J.A., Martyniuk, C.J., Crump, K., Xiong, H., Zhao, E., Popesku, J., Anisman, H., Cossins, A.R., Xia, X., Trudeau, V.L., 2008. Effects of fluoxetine on the reproductive axis of female goldfish (*Carassius auratus*). Physiol Genomics 35, 273-282.

Mennigen, J.A., Sassine, J., Trudeau, V.L., Moon, T.W., 2010b. Waterborne fluoxetine disrupts feeding and energy metabolism in the goldfish *Carassius auratus*. Aquat Toxicl 100, 128-137.

Mennigen, J.A., Stroud, P., Zamora, J.M., Moon, T.W., Trudeau, V.L., 2011. Pharmaceuticals as neuroendocrine disruptors: lessons learned from fish on Prozac. J Toxicol Environ Health B 14, 387-412.

Metcalfe, C.D., Chu, S., Judt, C., Li, H., Oakes, K.D., Servos, M.R., Andrews, D.M., 2010. Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. Environ Toxicol Chem 29, 79-89.

Metcalfe, C.D., Miao, X.S., Koenig, B.G., Struger, J., 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ Toxicol Chem 22, 2881-2889.

Miao, X.-S., Koenig, B.G., Metcalfe, C.D., 2002. Analysis of acidic drugs in the effluents of sewage treatment plants using liquid chromatography–electrospray ionization tandem mass spectrometry. J Chromatogr A 952, 139-147.

Mimeault, C., Woodhouse, A., Miao, X.-S., Metcalfe, C., Moon, T., Trudeau, V., 2005. The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish *Carassius auratus*. Aquat Toxicol 73, 44-54.

Mommsen, T.P., Walsh, P.J., Perry, S.F., Moon, T.W., 1988. Interactive effects of catecholamines and hypercapnia on glucose production in isolated trout hepatocytes. Gen Comp Endocr 70, 63-73.

Morgan, D.J., Okeke, I.N., Laxminarayan, R., Perencevich, E.N., Weisenberg, S., 2011. Non-prescription antimicrobial use worldwide: a systematic review. The Lancet infectious diseases 11, 692-701.

Nassef, M., Kim, S.G., Seki, M., Kang, I.J., Hano, T., Shimasaki, Y., Oshima, Y., 2010a. In *ovo* nanoinjection of triclosan, diclofenac and carbamazepine affects embryonic development of medaka fish (*Oryzias latipes*). Chemosphere 79, 966-973.

Nassef, M., Matsumoto, S., Seki, M., Khalil, F., Kang, I.J., Shimasaki, Y., Oshima, Y., Honjo, T., 2010b. Acute effects of triclosan, diclofenac and carbamazepine on feeding performance of Japanese medaka fish (*Oryzias latipes*). Chemosphere 80, 1095-1100.

Nickerson, J.G., Dugan, S.G., Drouin, G., Moon, T.W., 2001. A putative β2-adrenoceptor from the rainbow trout (*Oncorhynuchus mykiss*). Eur J Biochem 268, 6465-6472.

Nickerson, J.G., Dugan, S.G., Drouin, G., Perry, S.F., Moon, T.W., 2003. Activity of the unique β-adrenergic Na+/H+ exchanger in trout erythrocytes is controlled by a novel β3-AR subtype. Am J Phsyiol - Reg I 285, R526-R535.

Nunes, B., Gaio, A., Carvalho, F., Guilhermino, L., 2008. Behaviour and biomarkers of oxidative stress in *Gambusia holbrooki* after acute exposure to widely used pharmaceuticals and a detergent. Ecotox Environ Safe 71, 341-354.

Oliveira, R., Domingues, I., Grisolia, C.K., Soares, A.M., 2009. Effects of triclosan on zebrafish early-life
stages and adults. Environ Sci Poll Res 16, 679-688.

Owen, S.F., Giltrow, E., Huggett, D.B., Hutchinson, T.H., Saye, J., Winter, M.J., Sumpter, J.P., 2007. Comparative physiology, pharmacology and toxicology of β-blockers: mammals versus fish. Aquat Toxicol 82, 145-162.

Owen, S.F., Huggett, D.B., Hutchinson, T.H., Hetheridge, M.J., Kinter, L.B., Ericson, J.F., Sumpter, J.P.,
2009. Uptake of propranolol, a cardiovascular pharmaceutical, from water into fish plasma and its effects on growth and organ biometry. Aquat Toxicol 93, 217-224.

Painter, M.M., Buerkley, M.A., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., Furlong, E.T., Schultz, M.M., Schoenfuss, H.L., 2009. Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows (*Pimephales promelas*). Environ Toxicol Chem 28, 2677-2684.

Pedersen, J.A., Soliman, M., Suffet, I., 2005. Human pharmaceuticals, hormones, and personal care product ingredients in runoff from agricultural fields irrigated with treated wastewater. J Agri Food Chem 53, 1625-1632.

Peng, H.-C., Wang, Y.-H., Wen, C.-C., Wang, W.-H., Cheng, C.-C., Chen, Y.-H., 2010. Nephrotoxicity assessments of acetaminophen during zebrafish embryogenesis. Comp Biochem Phys C 151, 480-486.

Perreault, H.A., Semsar, K., Godwin, J., 2003. Fluoxetine treatment decreases territorial aggression in a coral reef fish. Physiol Behav 79, 719-724.

Poté, J., Ceccherini, M.T., Van, V.T., Rosselli, W., Wildi, W., Simonet, P., Vogel, T.M., 2003. Fate and transport of antibiotic resistance genes in saturated soil columns. Eur J Soil Biol 39, 65-71.

Prášková, E., Štěpánová, S., Chromcová, L., Plhalová, L., Voslářová, E., Pištěková, V., Prokeš, M., Svobodová, Z., 2013. The effects of subchronic exposure to ketoprofen on early developmental stages of common carp. Acta Veterinaria Brno 82, 343-347.

Prindiville, J.S., Mennigen, J.A., Zamora, J.M., Moon, T.W., Weber, J.-M., 2011. The fibrate drug gemfibrozil disrupts lipoprotein metabolism in rainbow trout. Toxicol App Pharm 251, 201-208.

Radjenović, J., Petrović, M., Barceló, D., 2009. Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. Water Res 43, 831-841.

Raldúa, D., André, M., Babin, P.J., 2008. Clofibrate and gemfibrozil induce an embryonic malabsorption syndrome in zebrafish. Toxicol App Pharm 228, 301-314.

Raut, S.A., Angus, R.A., 2010. Triclosan has endocrine-disrupting effects in male western mosquitofish, *Gambusia affinis*. Environ Toxicol Chem 29, 1287-1291.

Roberts, S.B., Langenau, D.M., Goetz, F.W., 2000. Cloning and characterization of prostaglandin endoperoxide synthase-1 and-2 from the brook trout ovary. Mol Cell Endocrin 160, 89-97.

Ruhoy, I.S., Daughton, C.G., 2007. Types and quantities of leftover drugs entering the environment via disposal to sewage—revealed by coroner records. Sci Total Environ 388, 137-148.

Runnalls, T.J., Hala, D.N., Sumpter, J.P., 2007. Preliminary studies into the effects of the human pharmaceutical Clofibric acid on sperm parameters in adult Fathead minnow. Aquat Toxicol 84, 111-118.

Sanderson, H., Brain, R.A., Johnson, D.J., Wilson, C.J., Solomon, K.R., 2004. Toxicity classification and evaluation of four pharmaceuticals classes: antibiotics, antineoplastics, cardiovascular, and sex hormones. Toxicology 203, 27-40.

Sattelberger, R., 1999. Residues of Pharmaceutical substances in the environment. Wien: Umweltbundesamt GmbH, 510-516.

Schlüter, A., Szczepanowski, R., Pühler, A., Top, E.M., 2007. Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. Microbiol Rev 31, 449-477.

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., Negele, R., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part I: histopathological alterations and bioaccumulation in rainbow trout. Aquat Toxicol 68, 141-150.

Schwartz, T., Kohnen, W., Jansen, B., Obst, U., 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS MicroBiol Ecol 43, 325-335.

Sedlak, D.L., Pinkston, K.E., 2011. Factors affecting the concentrations of pharmaceuticals released to the aquatic environment. J Contemp Water Res Edu 120, 7.

Skolness, S.Y., Durhan, E.J., Jensen, K.M., Kahl, M.D., Makynen, E.A., Villeneuve, D.L., Ankley, G.T., 2012. Effects of gemfibrozil on lipid metabolism, steroidogenesis, and reproduction in the fathead minnow (*Pimephales promelas*). Environ Toxicol Chem 31, 2615-2624.

Snieszko, S., Friddle, S., 1951. Tissue levels of various sulfonamides in trout. Trans Amer Fish Soc 80, 240-250.

Spencer, C.M., Barradell, L.B., 1996. Gemfibrozil. A reappraisal of its pharmacological properties and place in the management of dyslipidaemia. Drugs 51, 982-1018.

Stackelberg, P.E., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Henderson, A.K., Reissman, D.B., 2004. Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water-treatment plant. Sci Total Environ 329, 99-113.

Stanley, J.K., Ramirez, A.J., Chambliss, C.K., Brooks, B.W., 2007. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. Chemosphere 69, 9-16.

Stepanova, S., Praskova, E., Chromcova, L., Plhalova, L., Prokes, M., Blahova, J., Svobodova, Z., 2013. The effects of diclofenac on early life stages of common carp (*Cyprinus carpio*). Environ Toxicol Phar 35, 454-460.

Stumm-Zollinger, E., Fair, G.M., 1965. Biodegradation of steroid hormones. J Water Poll Cont Fed, 1506-1510.

Sumpter, J.P., 2007. Environmental effects of human pharmaceuticals. Drug Inf J 41, 143-147.

Sussman, N., 2003. SNRIs versus SSRIs: mechanisms of action in treating depression and painful physical symptoms. Prim Care Companion J Clin Psychiatry 5, 19-26.

Ternes, T., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res 32, 3245-3260.

Tixier, C., Singer, H.P., Oellers, S., Muller, S.R., 2003. Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters. Environ Sci Technol 37, 1061-1068.

Triebskorn, R., Casper, H., Scheil, V., Schwaiger, J., 2007. Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). Anal Bioanal Chem 387, 1405-1416.

Trudeau, V.L., Kah, O., Bourguignon, J.-P., 2011. Neuroendocrine disruption: the emerging concept. J Toxicol Envrion Health B 14, 267-269.

Trudeau, V.L., Metcalfe, C.D., Mimeault, C., Moon, T.W., 2005. Pharmaceuticals in the environment: Drugged fish? Biochem Mol Biol Fish 6, 475-493.

Van den Brandhof, E.-J., Montforts, M., 2010. Fish embryo toxicity of carbamazepine, diclofenac and metoprolol. Ecotox Environ Safe 73, 1862-1866.

Vollmer, G., 2010. Disposal of Pharmaceutical Waste in Households–A European Survey, Green and Sustainable Pharmacy. Springer, pp. 165-178.

Watkinson, A., Murby, E., Kolpin, D., Costanzo, S., 2009. The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. Sci Total Environ 407, 2711-2723.

Waye, A., Trudeau, V.L., 2011. Neuroendocrine disruption: more than hormones are upset. J Toxicol Environ Health B 14, 270-291.

Weber, M.A., 2005. The role of the new beta-blockers in treating cardiovascular disease. Am J Hypertens 18, 169S-176S.

Weinberger II, J., Klaper, R., 2014. Environmental concentrations of the selective serotonin reuptake inhibitor fluoxetine impact specific behaviors involved in reproduction, feeding and predator avoidance in the fish *Pimephales promelas* (fathead minnow). Aquat Toxicol 151, 77-83.

Winter, M.J., Lillicrap, A.D., Caunter, J.E., Schaffner, C., Alder, A.C., Ramil, M., Ternes, T.A., Giltrow, E., Sumpter, J.P., Hutchinson, T.H., 2008. Defining the chronic impacts of atenolol on embryo-larval development and reproduction in the fathead minnow (*Pimephales promelas*). Aquat Toxicol 86, 361-369.

Zikopoulos, B., Dermon, C.R., 2005. Comparative anatomy of α2 and β adrenoceptors in the adult and developing brain of the marine teleost the red porgy (*Pagrus pagrus, Sparidae*):[3H] clonidine and [3H] dihydroalprenolol quantitative autoradiography and receptor subtypes immunohistochemistry. J Comp Neuro 489, 217-240.

Zou, J., Neumann, N., Holland, J., Belosevic, M., Cunningham, C., Secombes, C., Rowley, A., 1999. Fish macrophages express a cyclo-oxygenase-2 homologue after activation. Biochem. J 340, 153-159.

Zuccato, E., Calamari, D., Natangelo, M., Fanelli, R., 2000. Presence of therapeutic drugs in the environment. The lancet 355, 1789-1790.

# CHAPTER 2

# CHRONIC, LOW CONCENTRATION EXPOSURE TO PHARMACEUTICALS IMPACTS MULTIPLE ORGAN SYSTEMS IN ZEBRAFISH (*Danio rerio*)

Reproduced with kind permission from Aquatic Toxicology.
[Galus, M., Kirischian, N., Higgins, S., Purdy, J., Chow, J., Rangaranjan, S., Li, H., Metcalfe, C., Wilson, J.Y., 2013. Aquat Toxicol 132-133, 200-211] © 2013

## ABSTRACT

Pharmaceuticals are found in both receiving and drinking water due to their persistent release in waste-water effluents, raising concerns for environmental and human health. Chronic, aqueous exposure of zebrafish (*Danio rerio*) to environmentally relevant concentrations of acetaminophen (ACE), venlafaxaine (VEN) (10 µg $L^{-1}$), carbamazepine (CBZ) and gemfibrozil (GEM) (0.5 and 10 µg $L^{-1}$) decreased reproductive output. Atretic oocytes and altered ovarian histology were seen in female zebrafish exposed to CBZ and GEM, suggesting a direct effect on oocyte development that may account for the reduced fecundity. Apoptosis within the theca and granulosa cells was identified in exposed female zebrafish with atretic oocytes by TUNEL positive staining. The incidence of follicular apoptosis was nearly 2 fold higher in exposed females than the controls. All compounds significantly altered kidney proximal tubule morphology but there was no difference in the incidence of apoptotic cells within the kidney between control and exposed in either males or females. Liver histology was altered by ACE and GEM exposure. Parental exposure to pharmaceuticals did not increase developmental abnormalities, hatching success, or mortality in embryos. Yet, direct exposure of embryos to ACE increased developmental abnormalities and mortality; exposure to 0.5 µg $L^{-1}$ of all pharmaceuticals increased mortality. CBZ decreased plasma 11-ketotestosterone concentrations in males and females. Overall, these data suggest that low concentration, chronic exposure of fish to pharmaceuticals impacts fish development as well as multiple organ systems in adult fish, leading to effects on reproduction and histology of liver and kidney. These results are significant in understanding the consequences of chronic, low concentration pharmaceutical exposure to fish and suggest that exposed populations are at risk of negative impacts to reproduction and health.

## INTRODUCTION

Studies have documented pharmaceutical and personal care products (PPCPs) in the aquatic environment at ng – μg $L^{-1}$ concentrations (Metcalfe et al., 2003) as well as micro-contamination of drinking water (Benotti et al., 2009). PPCPs enter the environment in waste-water effluent and release rates are expected to rise with increased use and aging demographics. These compounds are diverse in terms of mode of action and environmental exposure to PPCPs involves complex mixtures, yet we do not understand if PPCPs from different compound classes can impact similar biological endpoints or organ systems. PPCPs are potent, biologically active compounds suggesting that chronic, low concentration exposure of aquatic organisms and human populations may be of concern. Studies which focus on chronic, low concentration exposures will be most useful to determining possible effects of these compounds in the environment.

Although environmental concentrations are below human therapeutic doses, the persistent release of PPCPs may lead to chronic effects at low concentration. The lack of chronic toxicity data for PPCPs hinders our ability to predict effects. A notable exception is estrogens [e.g. 17α-ethynylestradiol (EE2)], where numerous studies have shown that environmentally relevant concentrations of estrogens are detrimental to fish (Lange et al., 2009). 5 - 6 ng $L^{-1}$ EE2 in an experimental lake feminized male fathead minnows *(Pimephales promelas)* and altered oogenesis in females, which led to a startling population crash within 2 years (Kidd et al., 2007).

Our study has focused on four PPCPs, from different chemical classes, that are commonly found in waste-water effluent and receiving waters (Metcalfe et al., 2003):

acetaminophen (ACE), gemfibrozil (GEM), venlafaxine (VEN), and carbamazepine (CBZ). ACE, a common analgesic and anti-inflammatory, inhibits cyclooxygenase (COX) activity (Hinz et al., 2008). Zebrafish exposed to COX inhibitors (10 - 100 μg $L^{-1}$ ibuprofen or ACE) have shown several developmental effects, including decreased hatching and growth rates, increased rates of spinal deformations, and increased mortality (10 – 100 μg $L^{-1}$) (David and Pancharatna, 2009a). Zebrafish exposed to 100 μg $L^{-1}$ indomethacin had reduced spawning rates and clutch sizes (Lister and Van Der Kraak, 2008). GEM, a fibrate lipid regulator and peroxisome proliferator (5000 μg$L^{-1}$), caused embryonic malabsorption syndrome in zebrafish (Raldua et al., 2008). Goldfish *(Carassius auratus)* exposed to GEM (1.5 μg $L^{-1}$) had decreased plasma testosterone (Mimeault et al., 2005). CBZ, used in the treatment of epilepsy and bipolar disorder, increased mortality and delayed development in Japanese medaka (*Oryzia latipeus*) injected with 0.012 μg CBZ $egg^{-1}$ (Nassef et al., 2010). CBZ caused severe spinal deformations resulting in an upward curvature of the tail (29,625 μg $L^{-1}$) (Weigt et al., 2011) and growth retardation (>30,600 μg $L^{-1}$) (van den Brandhof and Montforts, 2010) in zebrafish.

VEN is a heavily prescribed selective serotonin and noradrenergic reuptake inhibitor (SNRI) used in the treatment of depression (Beique et al., 1998). In fish, serotonin is known to affect behavior, stress responses, and food intake, as well as play a role in reproduction (Lepage et al., 2005; Ruibal et al., 2002). The effects of fluoxetine, a selective serotonin, but not epinephrine, reuptake inhibitor, have been heavily investigated in the goldfish, *C. auratus*. Environmental concentrations of fluoxetine have detrimental effects on feeding (Mennigen et al., 2009), behavior, and reproduction (Mennigen et al., 2008).

Goldfish exposed to 54 μg $L^{-1}$ fluoxetine for 14 days showed significant declines in basal milt production (Mennigen et al., 2010). Furthermore, fluoxetine significantly decreased both plasma concentrations of testosterone and mRNA expression of follicle-stimulating hormone and isotocin (the hormone homolog of oxytocin in fish), suggesting a disruption in signaling along the hypothalamus-pituitary-gonadal axis was likely responsible for lower milt production (Mennigen et al., 2010).

Several studies suggest PPCPs disrupt reproduction and development, yet most concentrations examined were well above environmentally relevant concentrations and the exposures acute. Whether environmentally relevant concentrations of PPCPs impact fish reproduction and development is unclear. The present study was designed to investigate whether chronic, low concentration exposure to four common PPCPs with different modes of action causes negative impacts in fish and whether common effects could be documented despite their differences. Using zebrafish, we have examined the reproductive (egg production and viability), developmental (mortality, abnormality and hatching rates), and histological effects of ACE, CBZ, GEM and VEN. These data are important for assessing whether there are common endpoints for mechanistically diverse PPCPs that may be used in studies of mixtures and field exposed fish. These data are important for understanding the risk of PPCPs to aquatic organisms and the possibility that persistent exposure to low concentration pharmaceuticals may be of concern.

## METHODS

*Fish Care*

Wild-type, adult zebrafish (DAP International, Canada) were housed at 4 fish $L^{-1}$ and 1:1 sex ratio in a recirculating system (28°C, pH 7-8, dissolved oxygen ≥87% and conductivity 470-455 μS) with ≥10% daily renewal. Aquaria water was made from distilled water supplemented with sodium bicarbonate and sea salts (Instant Ocean, Spectrum Brands, USA) to achieve the conditions above using an automatic dosing system. Fish were fed twice with a commercial food (Tetramin Tropical Flakes, Tetra, USA) and once with live, adult *Artemia* (GSL Brine Shrimp, USA) each day. Zebrafish were kept on a 14:10 hours (light:dark) light cycle. Fish were maintained in our facility for a minimum of one month prior to the onset of the experiment and breeding was initially monitored on a weekly basis. Fish were sorted to ensure a 1:1 sex ratio, based on a visual external examination prior to the first daily feeding and reproductive output was determined 3-5 times per week for a minimum of 2 weeks prior to the experiment. Embryo production was compared across tanks and only fish from tanks with equivalent reproductive output were included in the experiment. Tanks were randomly assigned a treatment and their fish moved to exposure tanks 3 days prior to the start of the experiment to allow acclimatization. All animal holding, breeding, and experimentation were performed in accordance to McMaster University's animal care policies and under an approved animal use protocol.

*Compounds Tested*

PPCPs (Sigma Aldrich, Canada) were dissolved in either nano-pure water (ACE and VEN) or dimethyl sulfoxide (GEM and CBZ). The final nominal concentration of each

PPCP in exposure tanks was 0 (Control), 0.5 (Low) or 10 (High) μg $L^{-1}$. The control and exposure tanks for GEM and CBZ had a final concentration of DMSO at 0.004%.

*Adult Zebrafish Exposures*

Triplicate tanks housed 50 (Experiment I) or 30 (Experiment II) zebrafish, with a sex ratio of 1:1, a density of 4 fish $L^{-1}$, in distilled water with 12 mg $L^{-1}$ sodium bicarbonate and 60 mg $L^{-1}$ sea salts (Instant Ocean, Spectrum Brands, USA); water chemistry was not different from the holding tanks. Tanks were recirculating (28 - 29°C and pH of 7.0 - 7.7) with a 90% renewal every 3 days, for six weeks. Weekly measurements of pH, dissolved oxygen, conductivity, nitrate, nitrite, ammonia, alkalinity, and general hardness were conducted. Zebrafish were fed as described above. The mean weight of control, low and high concentration fish was 431 ± 44, 445 ± 51, 443 ± 54 mg for males and 518 ± 76, 514 ± 52, 516 ± 60 mg for females. The mean weight was not different across treatment groups for either gender.

*Embryonic Zebrafish Exposures*

Fertilized embryos from unexposed parents were collected within 1.5 hours of first light from holding tanks, mixed and randomly placed individually in 48 well plates in E3 embryo-rearing media (1 mL $well^{-1}$; NaCl 5 μg $L^{-1}$, KCl 0.17 μg $L^{-1}$, $CaCl_2$ 0.33 μg $L^{-1}$ and $MgSO_4$ 0.33 μg $L^{-1}$). Embryos were exposed with appropriate PPCP at 0 (Control), 0.5 (Low) or 10 (High) μg $L^{-1}$. At least one 48 well control plate was followed for each embryo collection to determine background mortality in the clutch of embryos. Mortality can be variable across groups of embryos; embryos from unexposed parents typically exhibit less than 10% mortality in our facility. Data from control and treated embryos were excluded

when mortality in control plates was greater than 10%. The control and exposure plates for GEM and CBZ had a final concentration of DMSO at 0.004%; DMSO concentrations did not elevate mortality above background (i.e. compared to unexposed). Embryos were incubated at 28.5°C and observed until 72 hours post fertilization (hpf). Samples sizes were N=96 (ACE and GEM) and N=240 (CBZ and VEN) per treatment group.

*Water Sample Analyses*

During the third week of adult exposures, 10 mL of tank water was sampled at 10 min, and 24, 48, and 72 hrs after water change-outs. Water samples were covered in foil and stored at 4°C for no more than 48 hrs prior to extraction. Extractions were carried out on a Visiprep solid phase extraction (SPE) manifold (Supelco, USA) using Oasis HLB (ACE, GEM, and CBZ; Metcalfe et al., 2003) or Oasis MCX (VEN) SPE cartridges (Waters, Canada). ACE, GEM, and VEN samples were acidified to pH 2.0 with 3.5M $H_2SO_4$. CBZ samples were adjusted to pH 7.5 with either 1M NaOH or 3.5M $H_2SO_4$. Samples were spiked with 1 μg $L^{-1}$ of their respective surrogate standard. Cartridges were sequentially pre-conditioned with 6 mL of acetone and methanol and washed with HPLC grade water adjusted to pH 7.0 with 1.0M NaOH (HLB) or adjusted to pH 2.0 with 3.5M $H_2SO_4$ (MCX). Samples were aspirated at a rate of 10 mL $min^{-1}$. HLB cartridges were washed with 3 mL 1% $CH_2O_2$ and 3 mL 1% $NH_4OH$. Samples were eluted with 2 x 2% $NH_4OH$ in methanol and $CH_3OH$, allowing each 3 mL aliquot to incubate for 10 min. VEN and CBZ samples were eluted directly after the initial pass through the column. Samples were eluted with 3 x 3 mL 5% ($NH_4OH$) in methanol (v/v, VEN) or 3 x 3 mL $CH_3OH$ (CBZ).

PPCP concentrations were analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS) using methods adapted from Miao et al., (2002a; 2002b). In brief, 20 µL extract was analyzed on an Alliance 2695 liquid chromatograph (Waters, Milford, MA, USA) with a Genesis $C_{18}$ column (50 x 2.1 mm internal diameter, 3 µm particle size; Jones Chromatography, Hengoed, Mid Glamorgan, UK). The mobile phases were acetonitrile (A) and 20 mM ammonium acetate (B), operated with gradient elution at a flow rate of 0.3 mL $min^{-1}$. Mass spectrometry was performed using a Quattro LC tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Z-Spray electrospray ionization source. The LC-MS/MS system was operated in negative ionization for ACE and GEM, and positive ionization for CBZ. VEN extracts were analyzed by LC-MS/MS using an Agilent 1100 HPLC coupled to an Applied Biosystem Q-Trap tandem mass spectrometer fitted with an atmospheric pressure chemical ionization (APCI) source. (MDS Sciex, Concord, ON, Canada). The LCAPCI-MS/MS system was performed in positive ion mode. Chromatographic separation was achieved with a Genesis $C_{18}$ column (150 × 2.1 mm internal diameter, 4 µm particle size; Chromatographic Specialties) with a pre-column of the same packing material (4 mm x 2.0 mm; Phenomenex, Torrance, CA, USA). Both LC-MS/MS systems were operated in positive or negative ionization mode using multiple reaction monitoring (MRM) with one precursor-product ion transition for each analyte.

Quantification was performed using an internal standard method with a five-point calibration curve spanning the range of anticipated analyte concentrations in the samples,

using a weighted (1/concentration) linear regression. The limits of detection and limits of quantification were all below 0.003 µg $L^{-1}$ and 0.009 µg $L^{-1}$, respectively.

*Reproductive and Developmental Endpoints*

During adult exposure experiments (Experiment I and II), embryos were collected daily in a mesh covered plastic tray for 1.5 hours after first light and placed in E3 media at 28.5°C. All eggs were counted and observed at 6 hpf; embryos that were alive and actively dividing were considered viable embryos. Unfertilized eggs were included in counts of total eggs ovulated, those dead at 6 hpf were included in the count of total embryos. Reproductive data was normalized by the numbers of females in the tank, as confirmed by the presence of ovaries at necropsy. During Experiment I, approximately 50 viable embryos $tank^{-1}$ $week^{-1}$ were observed at 6, 24, 48 and 72 hpf for mortality, hatching and common developmental abnormalities (spinal cord deformations, pericardial edema, yolk sac edema, and stunted growth).

Female zebrafish may produce a clutch (≤ 250 embryos) every few days. Thus, we had expected to collect more embryos in our control tanks (Experiment I, Figure 2.2). We monitored reproduction of the fish for a minimum of 4 weeks prior to the experiment and all tanks were consistently producing embryos. Our egg collection methods use an egg trap placed inside the tank (see above for details) that does not collect every embryo produced. Alternative methods of separating groups of fish from their eggs produced poor water quality (e.g. funnel collector, such as in Meinelt et al., 2001) or high mortality even in embryos from unexposed animals (e.g. mesh inserts, such as in Lister et al., 2009) when scaled up for the numbers of fish and length of experiment needed in this study. We

performed several experiments to determine that the reproductive data were consistent and repeatable. Breeding studies comparing tank (with egg traps) and pairwise (with tank inserts that collect nearly all eggs) breeding showed consistent, repeatable embryo production with different groups of unexposed fish. We modified the tank breedings to use 30 fish (but the same density of fish, sex ratio, water, and feeding). The modified conditions reduced the water column over the egg traps and the recirculating filter was shut off during breeding to maximize egg capture. Tank breedings produced only 7.4% (original) or 26% (modified) of embryos collected during pairwise breeding; yet the data was consistent over time and across replicate groups of fish (data not shown). We repeated the high concentration exposures for all pharmaceuticals using the modified tank breeding design (Experiment II; 15 males and 15 females, recirculating filter turned off; all other experimental design as described above for Experiment I). A schematic of the design of Experiment I and II; and the timing of endpoints for each experiment, is shown in Figure 2.1.

*Blood and Tissue Sampling*

After 6 weeks exposure to pharmaceuticals, zebrafish were submerged in ice water, blotted dry and weighed. An incision was made in the axial plane directly below the urogenital opening, severing the caudal artery. Blood was collected with a micro-capillary tube, pooled from 15 - 20 fish and centrifuged at 21,155 g for 12 minutes. Plasma supernatant was stored at -80°C. Fish were sacrificed by severing the spinal column and gender confirmed by visual inspection of the gonads. The final mean sex ratio between males and females, across all tanks and exposures was 1:1.2. The whole body (open body

cavity) was fixed in formalin for a minimum of 48 hr, rinsed with 50% ethanol for 30 minutes and stored in 70% ethanol until decalcified with Richard Allen De-cal (Fisher Scientific, Ottawa, ON) following the manufacturer's protocol. Decalcified samples were embedded in paraffin at the core histology suite at McMaster University.

*Histology*

Paraffin embedded whole zebrafish were serially sectioned in the parasaggital plane at 5µm, deparaffinized with Citrisolv (Fisher Scientific, Ottawa, ON) and rehydrated with 100 – 70% ethanol, following standard histology procedures. Parasaggital sections were stained in hematoxylin and eosin Y (Richard Allen Scientific, Kalamazoo, USA). Stained slides were examined for histopathological alterations in liver, kidney and gonad on a Zeiss Axiolab microscope (Carl Zeiss, Hallbergmoos, Germany). 3-5 sections, approximately 100 – 150 µm apart, were stained and examined individually to identify which sections had the largest cross section for screening each organ (gill, liver, kidney, gonad). At least 2 sections were examined in detail for each organ to determine histopathology scores and rule out staining artifacts and regional differences in staining. The slides were read blind (for treatment, sections from the same individual were known) by a single assessor (M. Galus). Histological changes were noted and confirmed by a second assessor (J. Wilson) on a limited set of samples, prior to unblinding the data. Select images from impacted animals were sent to external labs for confirmation of the histological changes observed.

The functional units used to identify histopathological changes amongst the liver and kidney as well as the scoring system used to rank the regressive changes were from Bernet el al. (1999). Oocyte atresia was identified based on significant hyperplasia and

hypertrophy amongst the granulosa cells and the degeneration of vitellogenin protein, often leading to large gaps between the basement membrane and cell body (Sema and Özlem, 2009).

*TUNEL Assay*

Apoptotic cell death was detected using terminal dideoxynucleotidyl-mediated dUTP nick end labeling (TUNEL), with peroxidase (POD), according to the manufacturer's instructions (Roche Diagnostics, Laval, QC, Canada). Briefly, paraffin sectioned slides had basal levels of peroxidase blocked by incubation with 3% $H_2O_2$ in MeOH (v/v) for 15 minutes at 23°C. After incubation, slides were rinsed with phosophate buffered saline (PBS) and the TUNEL mixture was placed on the slide and allowed to incubate for 60 minutes at 37°C. Slides were rinsed with PBS once more and incubated in POD converter solution for 30 minutes at 37°C before a final rinse with PBS. Slides were then incubated with diaminobenzidine (DAB) (Roche Diagnostics, Laval, QC, Canada) for 60 minutes and counterstained with hematoxylin and eosin (Richard Allen Scientific, Kalamazoo, USA) before being examined on a Zeiss Axiolab microscope (Carl Zeiss, Hallbergmoos, Germany). Apoptotic regions appear brown under light microscopy when incubated with the chromogenic substrate DAB. Identification of TUNEL-positive cells was done on the basis of presence or absences of the DAB chromogen. TUNEL staining was completed on samples that had previously been identified as having more severe histological effects (histological scores of 3 or 4), based on hematoxylin and eosin staining, and control samples from CBZ and VEN exposures. We were unable to complete TUNEL staining on any ACE and GEM samples as any remaining tissue, on the histological cassettes, would

not yield sections with the desired tissue of interest (kidney in males and females and ovary in females). Testes were present in sections from males stained for the TUNEL assay; thus we have also examined testis for TUNEL positive staining.

*Sex Steroid Hormones*

Plasma samples were extracted and assayed using a commercially available enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI, USA) for the concentrations of estradiol ($E_2$) and 11-keto testosterone (11-KT), as previously described (Bowley et al., 2010).

*Statistical Analysis*

Data were examined for normality using the Shapiro-Wilk's test. Cumulative viable embryo production was normalized by the numbers of females per tank, based on gonadal inspection at dissection. Means and variance were based on triplicate tanks and differences in means were determined using an analysis of co-variance (ANCOVA), assessing for embryo production on a per female basis, factoring in concentration and length of exposure. Data was analyzed using SAS statistical software (version 8.02; SAS Institute, Cary, North Carolina USA). The incidence of developmental abnormalities and mortality within larval exposures was analyzed by a one-way analysis of variance (ANOVA). Mortality, hatching, and developmental abnormalities were normalized to the number of embryos at 72 hpf. Histological data were analyzed using a student's *t* test within each exposure based on the percent incidence of histopathology and the mean pathological score. Plasma hormone concentrations were not normal and were analyzed using a Kruskal–Wallis one-way analysis of variance on ranks. Statistical analyses on

adults were completed within a single gender, comparing responses in control to exposed fish from males and females separately. All statistical tests, except those involving cumulative viable embryo production, were analyzed using SigmaPlot 11.0 (Systat Software). Following ANOVA, patterns of significant difference were determined using a Holm-Sidak post-hoc test. All data were expressed as mean ± standard error. The significance level was set at $P = 0.05$.

## RESULTS

### *Waterborne Pharmaceutical Concentrations*

The mean aqueous concentrations of PPCPs are given in Table S1. CBZ and GEM concentrations were close to the nominal concentrations. ACE (high concentration) and VEN (both concentrations) were 50% lower than the nominal concentration. The low ACE concentration was approximately double the nominal concentration (Table S1). As expected, concentrations were not stable over time and concentrations at 72 hours post water change out were less than those at all other time points. Only the high concentration tanks for ACE showed a significant loss of compound 24 hours after water change out. Concentrations of PPCPs in the control were low but detectable except for VEN. ACE concentrations in the control were similar to the low dose tank (Table S1).

### *Pharmaceutical impacts on reproduction*

In all exposures (Experiment I), reproductive output declined with PPCP exposure. Zebrafish had significantly reduced embryo production with exposure to both concentrations of CBZ and GEM and high concentration of ACE and VEN (Figure 2.2). The reproductive declines with high concentration PPCP exposure were consistent and

repeatable (Table 2.1). Using modified experimental methods to increase the embryo capture rate (see above for details), the number of viable embryos per female was approximately five times greater in both control and exposed groups, yet the percent decrease in embryo production with PPCP exposure was nearly identical to our original experiments and all pharmaceuticals caused a statistically significant decline in reproduction at the high concentration in both experiments (Table 2.1).

*Pharmaceutical impacts on development*

Parental PPCP exposure did not impact the survival, hatching success, and rate of developmental abnormalities in offspring. Yet, direct PPCP exposure of zebrafish embryos increased mortality at the low concentration of all PPCPs (Figure 2.3A). Exposure to ACE and GEM increased mortality at the high concentration. Interestingly, ACE exposure resulted in a level of mortality that was ~ 4.5 fold higher than with any other PPCP (Figure 2.3A). Surprisingly, high CBZ exposure caused a statistically significant decrease in mortality.

Embryonic zebrafish exposed to ACE showed 13.5 (low concentration) and 6.0 (high concentration) fold increases in developmental abnormalities, compared to controls (Figure 2.3B). This increase was driven by increased spinal deformation and pericardial edema. Spinal deformations were 6 and 3 fold higher in the low and high concentrations, respectively, over controls. These deformations included curved or bent spinal columns as well as truncations of the tail. Pericardial edema was 2 fold higher in the low concentration than in either control or high concentrations. Interestingly, multiple abnormalities, primarily pericardial edema and spinal deformations, were seen in some zebrafish exposed

to ACE but not in any control animals. GEM and CBZ had no effect on developmental abnormalities. VEN caused a 3 and 7 fold decrease in developmental abnormalities at the low and high concentration groups, respectively. For all compounds, greater than 99% of all embryos were hatched by 72 hpf.

*Organ Histology and TUNEL staining*

We examined 2 to 3 sections for histopathology using hematoxylin and eosin stained whole mounted fish. Exposure to all four PPCPs resulted in a significant increase in mean histopathological score and incidence of regressive changes in kidney tubule structure (Table S2). Control fish had normal kidney tubule morphology (Figure 2.4A), yet greater than 60% of exposed animals had regressive changes in the kidney tubules; the incidence was similar across concentration and gender. The severity of regressive changes increased with concentration (Figure 2.4B-D). The regressive changes included structural alterations to the tubule structure, alterations in nuclear size or density, plasma alterations, loss of staining integrity, and the presence of hyaline or hypertrophic/hyperplasic tissue. These changes were primarily located in the proximal tubules. Proteinaceous fluid accumulation, likely eosinophilic due to the presence of blood cells in some fluid, was found in regions surrounding the kidney tubules after PPCP exposure (Figure 2.4B) but was not visible in sections from control animals (Figure 2.4A). Those animals with high kidney histopathology scores (3 or 4) and control animals were screened for the presence of TUNEL-positive cells. However, there was no difference between controls and exposed males or females in the occurrence of apoptosis within the kidney.

Exposure to 0.5 and 10 µg $L^{-1}$ ACE and GEM, respectively, caused a significant increase in the incidence and severity of regressive changes in the liver in males (Table S3). Similarly, 0.5 and 10 µg $L^{-1}$ GEM and ACE, respectively, caused histological changes in the liver in females (Table S3). Both compounds caused a decrease in the amount of native glycogen stores as well as an increase in the size of hepatic nuclei, compared to control fish (Figure 2.5B).

All developmental stages of spermatogenesis, including spermatozoa, were present in all examined histological sections. Exposure to PPCPs had no observable effect on testis morphology (Table S4), nor was there any difference between controls and exposed animals in the occurrence of apoptosis within the testes, as determined by TUNEL positive cells.

Exposure to CBZ and GEM increased the occurrence of atreitic oocytes in females (Figure 2.5D, Table S4). Animals exposed to 0.5 µg $L^{-1}$ CBZ and GEM primarily had oocytes with irregularities in the somatic stromal tissue, whereas those exposed to 10 µg $L^{-1}$ GEM had a higher abundance of pre-vitellogenic oocytes, with only a few mature follicles and a large number of perinuclear oocytes. In adult female zebrafish, the incidence of apoptotic ovarian follicles was 1.82 times higher in PPCP exposed animals compared to controls. Ovarian follicles were judged to be apoptotic based on the criteria of TUNEL positive staining within the thecal and granulosa, as well as morphological changes within these layers (Sema and Özlem, 2009; Wood and Van Der Kraak, 2001). The TUNEL positive cells were limited to early vitellogenic oocytes (S1) and strictly contained within the somatic tissue in controls. On all control sections that showed S1 TUNEL positive

cells, there was no TUNEL positive staining in mature (S3/S4) follicles or within the theca and granulosa. All exposed females with TUNEL positive staining showed hypertrophic cells within the granulosa and enlarged zona radiata (Figure 2.6B). Separation of the granulosa from the basement membrane of the follicle was observed in 38% of exposed females (Figure 2.6B). TUNEL positive ooplasmic vesicles were observed in all exposed females with ovarian apoptosis.

*Sex Steroid Concentrations*

Mean plasma estradiol concentrations were similar amongst treatment groups for both males and females (Figure 2.7). Plasma 11-ketotestosterone (KT) levels were significantly decreased by CBZ exposure in both sexes (Figure 2.7). Male and females exposed to 0.5 µg $L^{-1}$ CBZ showed a 9.5 and 2.2 fold decline in circulating KT levels compared to controls.

## DISCUSSION

The implications of chronic, low concentration exposure of aquatic fauna and humans to environmental discharge of PPCPs remain largely unknown. Only estrogens are well studied in terms of environmental effects yet numerous PPCPs are found in the aquatic environment. Concentrations of PPCPs are typically in the ng - µg $L^{-1}$ range in the aquatic environment (Andreozzi et al., 2003; Benotti and Brownawell, 2007; Brun et al., 2006; Kolpin et al., 2002; Metcalfe et al., 2003; Tixier et al., 2003) and ng $L^{-1}$ concentrations in drinking water (Benotti et al., 2009). In this study, the four PPCP tested represent different pharmaceutical classes found in the environment at environmentally relevant concentrations; the high concentration was near the highest reported concentrations. Since

complex PPCP mixtures are found in the environment, many pharmaceuticals are present with similar modes of action and thus the high concentration is similar to the concentration for all PPCPs within a single drug class. The concentrations chosen were based on the range reported in the literature, although clearly at each site the concentrations of different PPCPs vary with pharmaceutical usage patterns, the size of the surrounding population, the specific wastewater treatment available, and discharge location. Thus, the concentrations are not meant to be an exact match to what is found at one wastewater treatment plant, or field site, but to represent concentrations that are reasonable and environmentally relevant. To our knowledge, this is the first study to show that chronic, low concentration PPCP exposure, with the exception of estrogens, have impacts on multiple organ systems in fish.

Analyses of the tank water confirm that the PPCPs were present throughout the time between water change outs (72 hours), although the dose declined with time (Table S1). While actual tank concentrations were less than the nominal concentrations for VEN, concentrations of ACE, CBZ and GEM were similar to nominal at 10 min post water change out. Surprisingly, ACE concentrations in the control tank were similar to the low dose suggesting that ACE exposure was not significantly higher in the low tank. Yet, significant effects were seen on kidney histology at the low dose suggesting that over the 6 week exposures, there was a difference in ACE exposure between control and low. As we only sampled one water change out in the 3$^{rd}$ week of exposure, all of the data for the control tank is from a single dose and represents only 3 days of the 42 day experiment.

*Reproductive Endpoints*

Chronic exposure of adult zebrafish to PPCPs (Figure 2.2, Table 2.1) decreased reproductive output. While number of viable embryos decreased with exposure, this reflects a reduction in embryos produced by the females, i.e. fecundity, and not decreased fertilization rates. Our results are consistent with other studies that show reproductive impacts from PPCPs and wastewater exposures in fish (Kidd et al., 2007; Lister et al., 2009). Previous studies have shown reproductive impacts with ACE (David and Pancharatna, 2009a, b) or the related COX inhibitor indomethacin (Lister and Van Der Kraak, 2008); GEM (Mimeault et al., 2005) or the related fibrate clofibric acid (Runnalls et al., 2007), and fluoxetine (Mennigen et al., 2010; Mennigen et al., 2008), an antidepressant with a similar, but not identical, mechanism of action to VEN. We know of no work examining the reproductive effects of CBZ on fish, yet CBZ has been shown to have negative effects on mammalian reproduction (de Oliva and Miraglia, 2009). Although all of the PPCPs are from different pharmaceutical classes, they appear to commonly impact the reproductive axis albeit likely through independent mechanisms of action.

ACE is a cyclooxygenase (COX) enzyme inhibitor. COX enzymes catalyze arachidonic acid into prostaglandins (PGs) (Marnett et al., 1999) and ACE exposure typically reduces PG levels in mammals. COX enzymes have been identified in zebrafish, have 65 – 73% sequence similarity to human homologs, and are inhibited by mammalian COX inhibitors (Grosser et al., 2002). In teleost fish, PGs have a key function in regulating reproduction, paracrine roles in stimulating male and female sexual behavior (Sorensen et al., 1988), ovulation (Mercure and Van Der Kraak, 1996), and estradiol production (Lister

and Van Der Kraak, 2008). Past studies have shown reproductive impacts of COX inhibitors, including ACE at concentrations >50 μg $L^{-1}$ (David and Pancharatna, 2009a, b; Lister and Van Der Kraak, 2008). We have demonstrated that chronic exposure at 10 μg $L^{-1}$ ACE impacts reproductive output in zebrafish.

CBZ stabilizes voltage gated sodium channels in the inactive state, reducing excitability of neurons. In vertebrates, the reproductive system is innervated by sympathetic and parasympathetic nerves (Seda et al., 2007) and reductions in neuron excitability may reduce neuronal stimulation to reproductive organs and gonadal steroids synthesis. Wistar rats exposed to 20 mg $kg^{-1}$ CBZ had lower plasma testosterone concentrations and reduced sperm quality (de Oliva and Miraglia, 2009). While we did not measure sperm quality, CBZ reduced KT, the predominant teleost androgen, suggesting the mode of action for this drug in fish may be similar to that of mammals. We found reductions in fecundity and not in fertilization rates of the embryos, suggesting that if sperm quality was impacted, sperm quantity and/or quality were still sufficient for effective fertilization of eggs. Sperm quality was not examined in this study and may need further investigation for understanding the impacts of CBZ exposure in fish.

GEM is a peroxisomal proliferator-activated receptor alpha (PPARα) ligand and activated PPAR upregulates peroxisome sensitive genes in mammals (Gervois et al., 2000). The peroxisomal oxidation system, including PPARα, exists in fish (Crockett and Sidell, 1993). Fathead minnows exposed to 0.01 – 1 mg $L^{-1}$ clofibric acid had impaired spermatogenesis and reduced sperm count (Runnalls et al., 2007). Interestingly, we did not observe any impacts on sperm morphology by GEM (Table S4), nor reduced

fertilization, but found impacts on oocytes (Table S4), specifically vitellogenin (VTG) content (Figure 2.5B). The lipid regulating properties of GEM may have impacted hepatic production of VTG. High levels of VTG are required for normal oocyte production and egg quality declines with reduced VTG (Brooks et al., 1997). Oocyte quality and size tend to decline and atretic oocyte production increases when pollutants accumulate within the oocyte (Brooks et al., 1997), which could explain our histological observations.

VEN inhibits the uptake of serotonin and norepinephrine at the presynaptic membrane, increasing neurotransmitter concentrations in the synaptic cleft. Studies have identified serotonin receptors in fish (De Lucchini et al., 2001) and the importance of serotonin in several physiological processes linked to behavior and reproduction (Lepage et al., 2005; Ruibal et al., 2002). Fluctuations in serotonin levels are correlated to reproductive phases in female fish (Hernandez-Rauda et al., 1999). Specifically, serotonin regulates gonadotropin II release, which stimulates gonadal steroid production. 54 μg $L^{-1}$ fluoxetine reduced plasma testosterone concentrations and basal milt production in male goldfish (Mennigen et al., 2010). Yet, VEN had no detectable impact on plasma 11-ketotestosterone levels (Figure 2.7). Caution must be used when interpreting our steroid hormone data because the need to pool many individuals caused a reduction in the numbers of samples available for these analyses. 32 μg $L^{-1}$ fluoxetine reduced egg production, ovarian E2 and ovarian follicle-stimulating and luteinizing hormone receptor expression in female zebrafish (Lister et al., 2009). In the present study, zebrafish exposed to 10 μg $L^{-1}$ VEN (Figure 2.2D) spawned fewer embryos. Although VEN did not alter plasma E2 levels (Figure 2.7) or cause gonadal histological changes, we did observe a higher abundance of

pre-vitellogenic oocytes in histological sections in females at 10 μg $L^{-1}$ (data not shown). High concentrations of norepinephrine stimulated pituitary gonadotropin release in female goldfish (Chang and Peter, 1984). Regardless of whether the reproductive effects of VEN are mediated by serotonin, norepinephrine, or both, it is mostly likely that VEN impacted the hypothalamus-pituitary-gonadal axis.

*Kidney Histology*

Teleost kidneys are primarily involved in pH, water balance and excretion of exogenous solutes. Freshwater fish are hyperosmotic to their environment and must eliminate large volumes of dilute urine and retain salt; fish have high urine flow and glomerular filtration rates (Perry, 1997; Perry et al., 2003). PPCP exposure resulted in regressive changes to the renal proximal tubule (Table S2, Figure 2.4), effects that may impact plasma concentrations of xenobiotics and homeostatic control of water, pH and ions. The renal proximal tubule is the segment of the nephron largely responsible for actively excreting xenobiotic compounds (Terlouw et al., 2001). Although it is uncertain how soon after exposure the regressive changes in kidney tubules occurred, rainbow trout and carp exposed to carbamazepine, clofibric acid, metoprolol, and diclofenac showed alterations of the proximal tubule as soon as 28 days post exposure (Triebskorn et al., 2007). These regressive changes may yield poor renal clearance and ultimately elevated body burden of pharmaceuticals during chronic exposure, perhaps explaining why we have seen effects at lower concentrations than previously reported for shorter exposure times. Further studies on pharmaceutical impacts on kidney function will require a larger fish species than zebrafish, preferably one where cannulation and collection of urine is possible.

*Embryonic Effects*

In all exposures, low concentration PPCPs elevated embryonic mortality (Figure 2.3). Interestingly, ACE elevated mortality compared to controls and all other exposures, yet the concentration response was not linear (Figure 2.3). ACE exposure alone caused an increase in developmental abnormalities, particularly pericardial edema and spinal deformation, both common abnormalities found at low rates in unexposed embryos. The embryonic effect of ACE may be mediated through COX enzymes. 68% of embryos, in both concentrations, died between 6-24 hpf, a time frame which encompasses gastrulation. COX genes are expressed during zebrafish embryogenesis and transcriptional blocking of COX-1 expression caused gastrulation arrest, an effect that was rescued with PG supplementation (Grosser et al., 2002). Interestingly, COX derived PGs are vital for normal segmentation and inhibition of zebrafish COX enzymes led to shortening of inter-somatic vessels (Cha et al., 2005; 2006), which could cause the onset of edema.

*Implications for environmental health*

The risks to aquatic species and human populations from chronic, low concentration PPCP exposure are unclear. Chronic exposure to environmentally relevant PPCP concentrations (Andreozzi et al., 2003; Benotti and Brownawell, 2007; Brun et al., 2006; Kolpin et al., 2002; Metcalfe et al., 2003; Tixier et al., 2003) impacts multiple organ systems in fish. Reproduction and development were negatively impacted in exposed fish, processes that are critical to maintenance of wild fish populations. All four PPCPs tested reduced the fecundity of zebrafish and caused histopathological changes in kidney. In addition, histopathological changes were seen in liver and ovaries with some of the PPCPs

tested. Plasma 11-KT levels were altered with CBZ exposure. Mortality of embryos was elevated at low concentration PPCP exposure and ACE exposure elevated developmental abnormalities. Reproductive output and kidney tubule histopathology appear to be common biological effects with multiple drug classes, suggesting these may be good endpoints for examination of mixtures and wastewater effluent exposures. This will require confirmation that the effects seen with individual pharmaceuticals are also found with exposures to PPCP mixtures and wastewater effluent. These biological endpoints may prove useful, for studies of both field and laboratory exposed fish, for determination of mechanisms of action of individual drugs and mixture toxicity in fish. Although environmental concentrations are low, our data suggests that with chronic exposures, low concentrations are sufficient to impact fish and that fish populations are at risk of adverse effects of pharmaceutical exposure.

## ACKNOWLEDGEMENTS

The authors wish to thank the members of the Wilson laboratory for help during sampling assistance; in particular we thank Lucas Bowley, James Harskamp, Patrick Schenck, Marcus Scornaienchi, Emily Smith, John Soleas, Shawn Parascandalo and Mika Yoshikawa. We thank Dr. Mark Servos for discussions regarding the histological changes in this study and for providing opinions on the histology observations and scoring system. Funding was provided by the Natural Sciences and Engineering Research Council of Canada in the form of a Strategic Project Grant to J.Y.W and C.D.M (No. 90340311), with the City of Hamilton, Water and Wastewater Division, as a partner agency. The Canadian Foundation for Innovation and Ontario Innovation Trust provided infrastructure support to J.Y.W.

**Table 2.1: Reproduction in zebrafish exposed to pharmaceuticals**. Zebrafish were exposed for 6 weeks to 0 (Ctrl), 0.5 (Low), or 10 (High) µg $L^{-1}$ of acetaminophen (ACE), carbamazepine (CBZ), gemfibrozil (GEM), or venlafaxine (VEN). Embryos were collected daily to determine reproductive output. For experiment I, 50 fish per tank were mated and embryos were collected with an egg trap with the filter running (see methods details). For experiment II, 30 fish per tank were mated and embryos were collected with an egg trap with the filter off. Numbers of embryos collected per female are shown for each concentration. The percent reduction in embryo produced per female from high concentration to control are shown for each experimental set up (% Reduction for High). All pharmaceuticals caused a statistically significant reduction in embryo production at the high concentration for both experiment I and II.

| Pharmaceutical | Experiment I | | | | Experiment II | | |
|---|---|---|---|---|---|---|---|
| | Ctrl | Low | High | % Reduction for High | Ctrl | High | % Reduction for High |
| **ACE** | 93 | 74 | 59 | 37 | 453 | 322 | 29 |
| **CBZ** | 130 | 85 | 77 | 41 | 257 | 188 | 27 |
| **GEM** | 104 | 62 | 44 | 58 | 257 | 144 | 44 |
| **VEN** | 117 | 127 | 48 | 59 | 453 | 227 | 50 |

**Figure 2.1: A schematic of the experimental design and timing of sampling.** A schematic representing the difference between Experiment I and II.

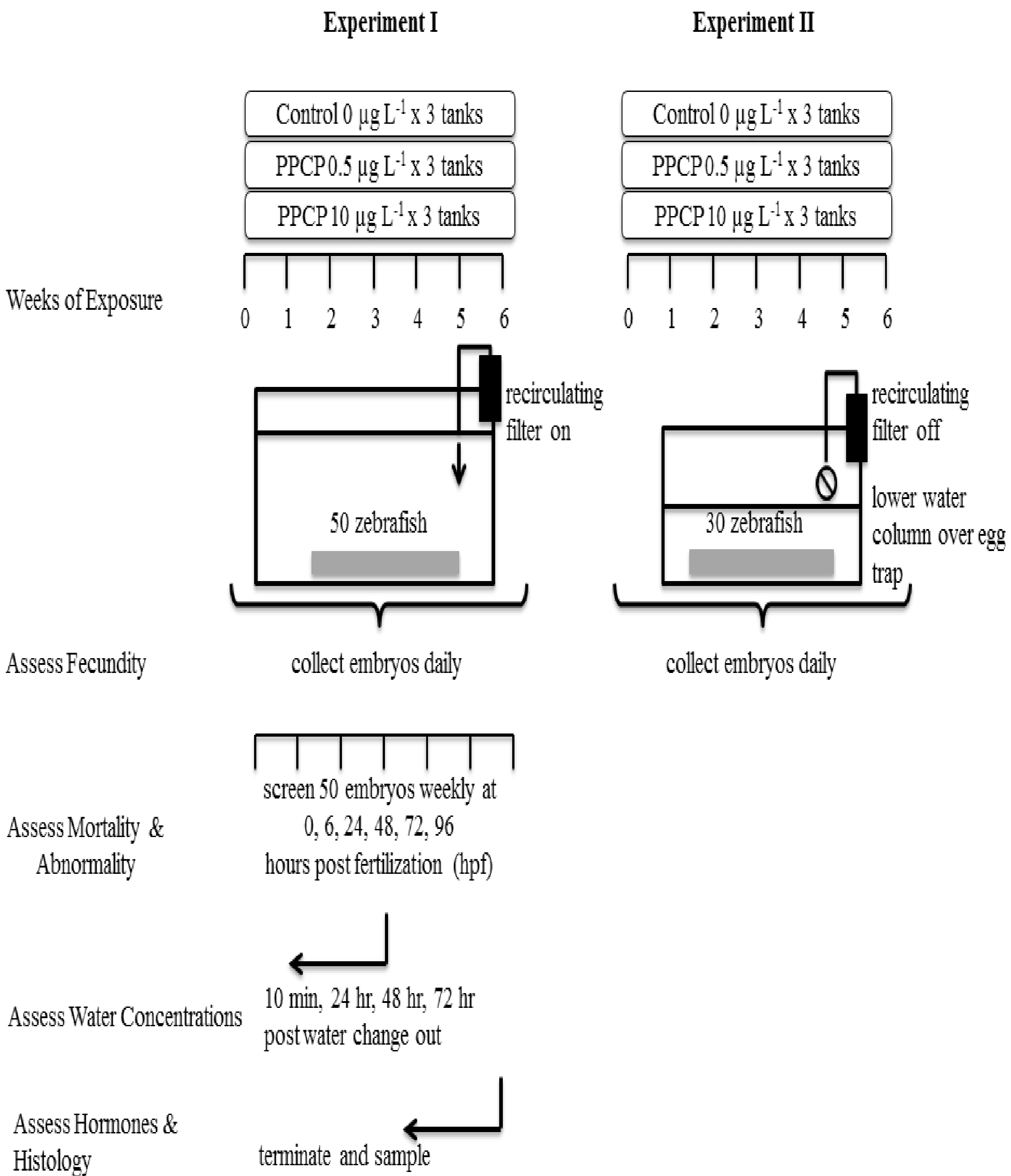

Experiment I
Experiment II
Control 0 µg L-1 x 3 tanks
PPCP 0.5 µg L-1 x 3 tanks
PPCP 10 µg L-1 x 3 tanks
Control 0 µg L-1 x 3 tanks
PPCP 0.5 µg L-1 x 3 tanks
PPCP 10 µg L-1 x 3 tanks
Weeks of Exposure
0 1 2 3 4 5 6
0 1 2 3 4 5 6
recirculating filter on
50 zebrafish
recirculating filter off
lower water column over egg trap
30 zebrafish
Assess Fecundity
collect embryos daily
collect embryos daily
Assess Mortality & Abnormality
screen 50 embryos weekly at 0, 6, 24, 48, 72, 96 hours post fertilization (hpf)
Assess Water Concentrations
10 min, 24 hr, 48 hr, 72 hr post water change out
Assess Hormones & Histology
terminate and sample

**Figure 2.2: Cumulative daily embryo production in zebrafish chronically exposed to pharmaceuticals**. Cumulative mean viable embryo production, per female, from triplicate tanks during 6 week exposures to 0 (control), 0.5 (low) and 10 (high) μg $L^{-1}$ of A) acetaminophen, B) carbamazepine, C) gemfibrozil and D) venlafaxine. * and † indicate significantly different from controls and high, respectively; $p < 0.05$.

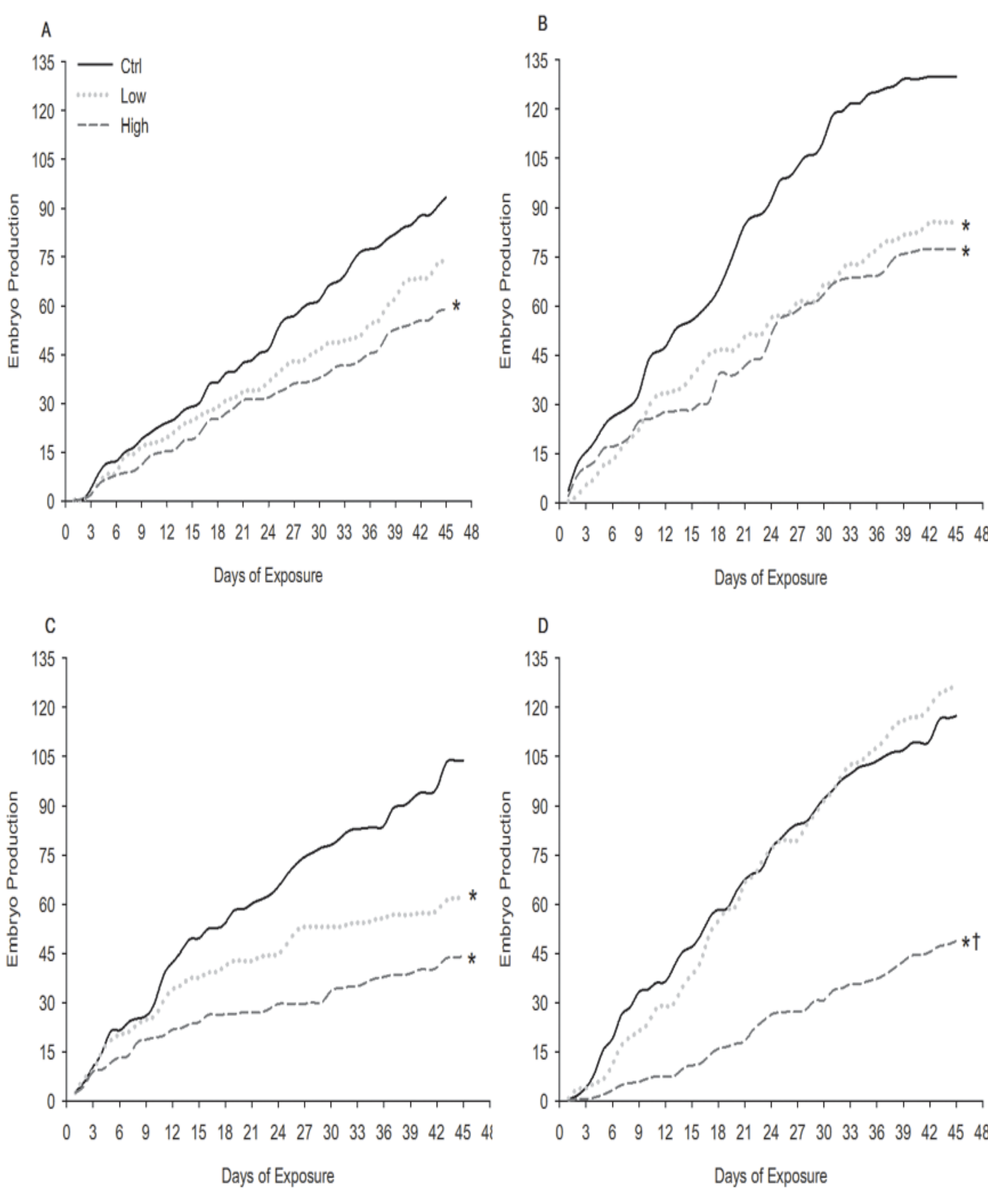

A
B
C
D
Ctrl
Low
High
Embryo Production
Days of Exposure
*
*†

**Figure 2.3: Total mortality and developmental abnormalities in zebrafish after embryonic exposure to pharmaceuticals**. Embryos were exposed to 0 (Ctrl), 0.5 (Low) and 10 (High) μg $L^{-1}$ acetaminophen (ACE), carbamazepine (CBZ), gemfibrozil (GEM) or venlafaxine (VEN) for 96 hpf. Total mortality (A) or abnormalities (B) were determined for each treatment group. N=96 (ACE and GEM) or N=240 (CBZ and VEN). Different letters denote statistically different ($p < 0.05$) groups within each exposure and not across exposures.

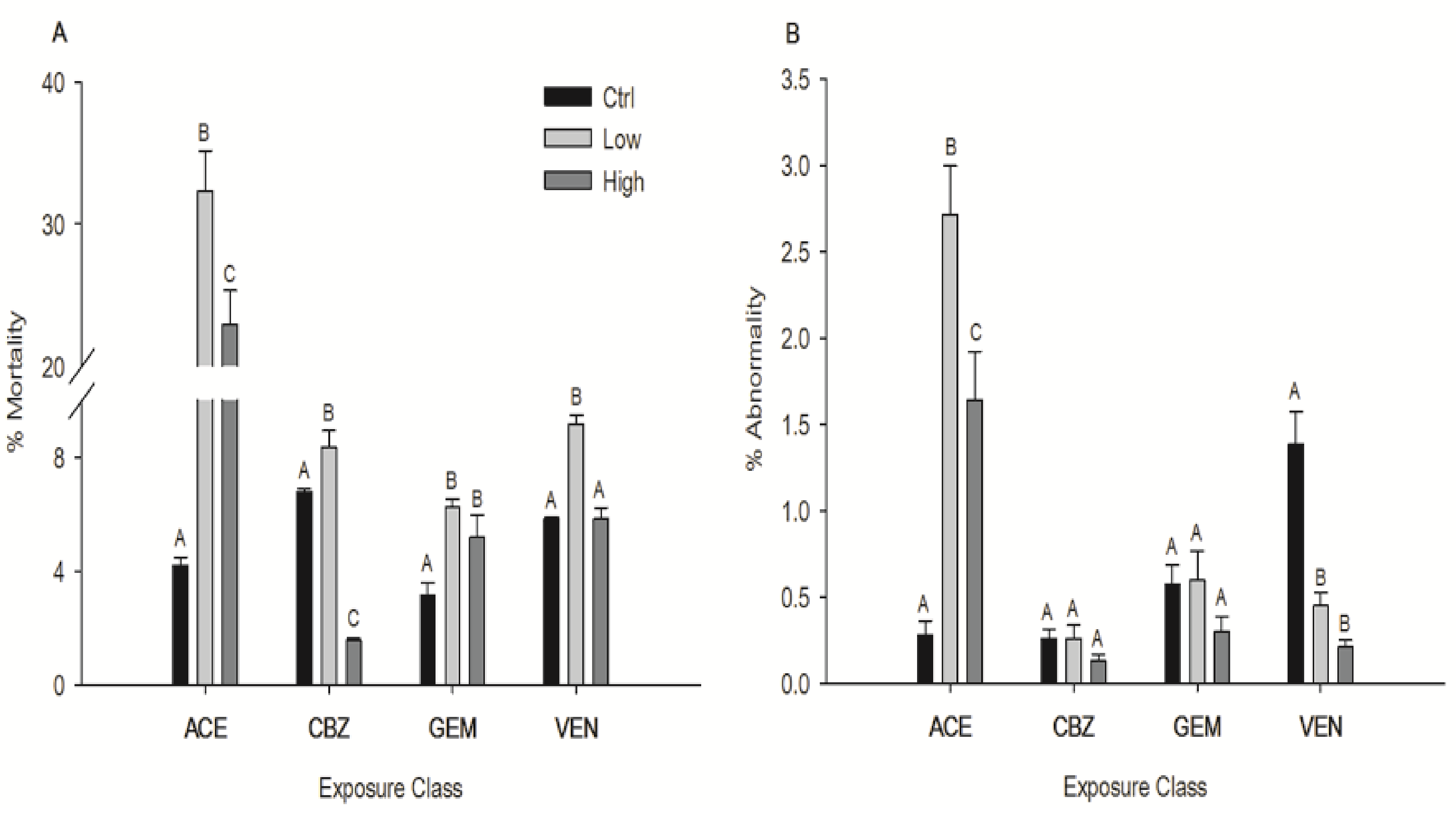

A
40
30
20
8
4
0
% Mortality
Ctrl
Low
High
ACE
CBZ
GEM
VEN
Exposure Class
B
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
% Abnormality
ACE
CBZ
GEM
VEN
Exposure Class

**Figure 2.4: Histological alterations in kidney after pharmaceutical exposure**. A) Normal kidney tubules, from a female zebrafish, showing central nuclei positioning within a completely stained cytoplasm and uniform nuclear area within a kidney tubule bundle (white arrows). B) Kidney tubules of a female zebrafish exposed to 10 µg $L^{-1}$ venlafaxine, showing cells with no apparent nuclei (black arrow), the early stages of cytoplasmic degradation (black arrow head) and small nuclei (open arrow head) (score 2 on histopathological index). Proteinaceous fluid is visible between the kidney tubules throughout this section (open arrow). C) Kidney tubules of a female zebrafish exposed to 10 µg $L^{-1}$ acetaminophen, showing progressed loss of cytoplasmic staining integrity (solid white and black arrow), non-uniform nuclei size, multi nuclei and small nuclear area (black arrow head), and complete loss of structural integrity amongst the tubule (score 3 on histopathological index). D) Kidney tubules of a female zebrafish exposed to 10 µg $L^{-1}$ acetaminophen, showing severe loss of cytoplasmic staining integrity (solid black arrow), sickle shaped nuclei (black arrow head), complete loss of cytoplasmic staining with non-central nuclei positioning (open arrow), complete breakdown of the native structure of the kidney tubule (open arrow) and eosinphilic fluid spanning the intertubal space (*) (score 4 on histopathological index). (H&E, 60x).

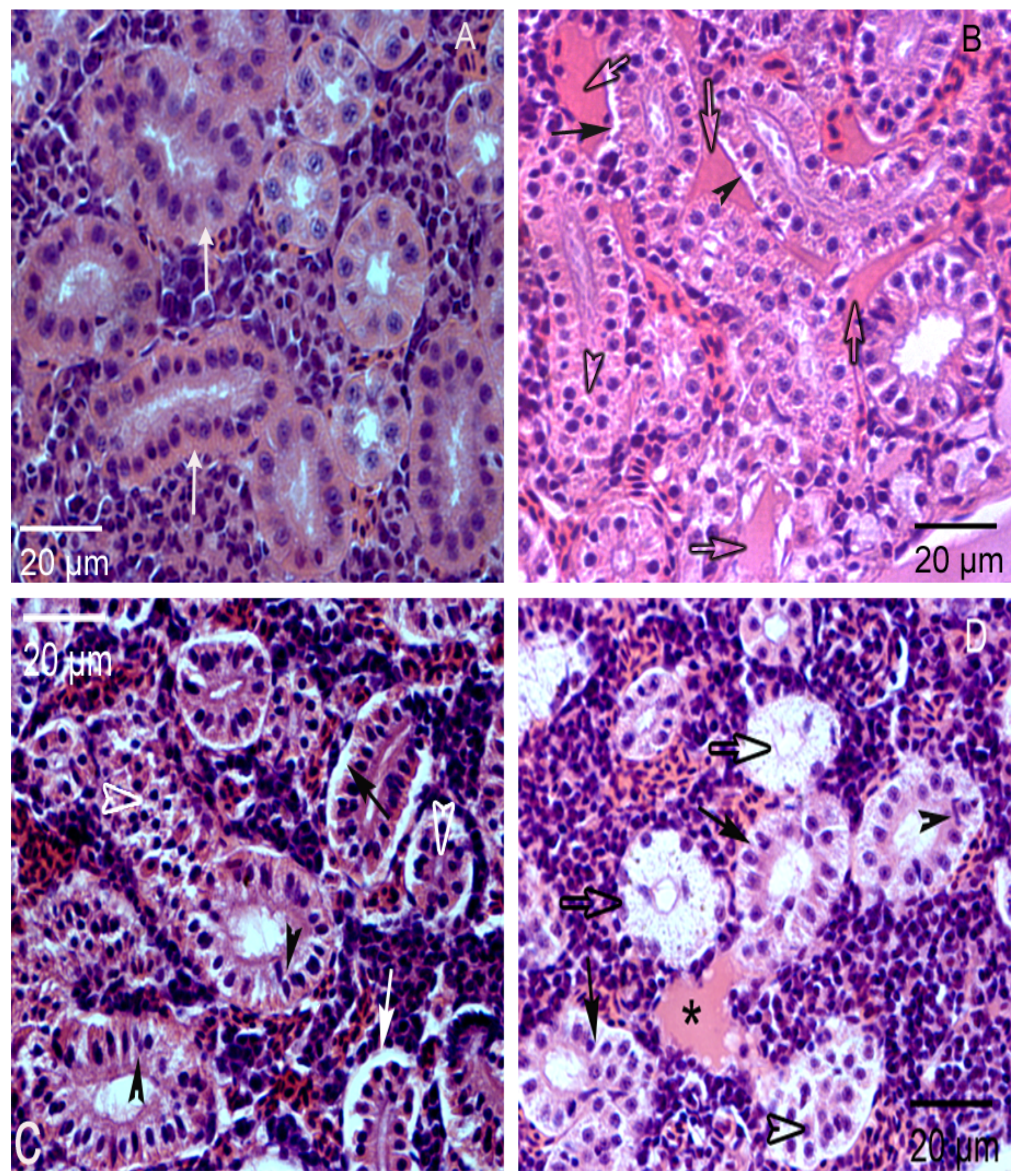

A
20 µm
B
20 µm
20 µm
C
D
*
20 µm

**Figure 2.5: Histological alterations in liver and ovary after pharmaceutical exposure**. A) A male zebrafish showing normal hepatic tissue with uniform hepatic nuclear area (black arrows). B) Hepatocytes of a male zebrafish exposed to10 µg $L^{-1}$ gemfibrozil, showing an increase in hepatic nuclear area (black arrow) and glycogen depletion (black arrow head) (H&E, 60x). C) Normal ovary at intermediate vitellogenic stages of development. Pre-vitellogenic oocytes (I and II) are mainly located within the periphery, whereas vitellogenic oocytes (III and IV) are located centrally within the ovarian tissue. D) Ovary of a female zebrafish exposed to 10 µg $L^{-1}$ carbamazepine. Arrows identify atreitic oocytes (H&E, 20x).

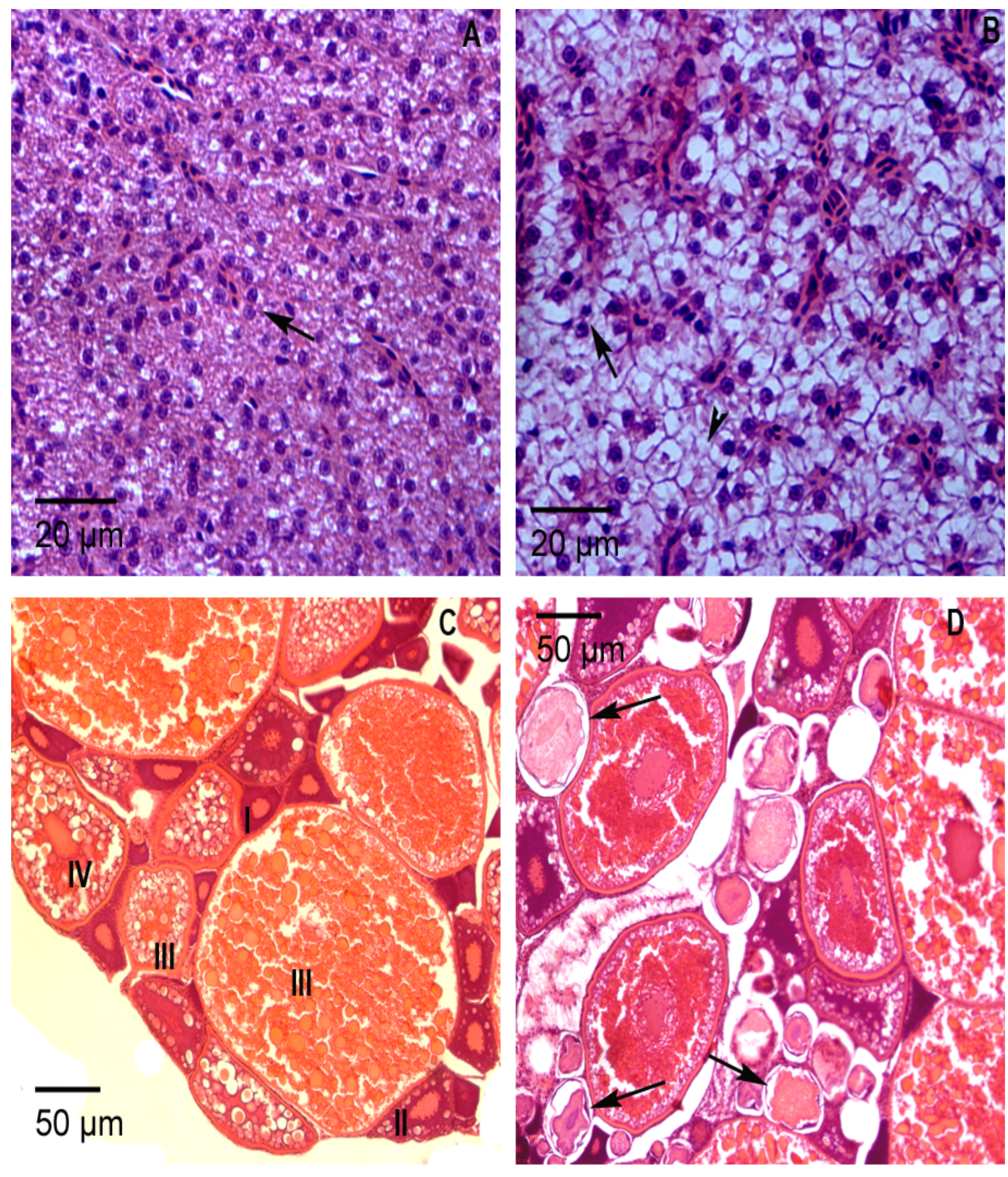

A
20 µm
B
20 µm
C
I
IV
III
III
II
50 µm
D
50 µm

**Figure 2.6: Apoptosis within the oocyte after pharmaceutical exposure.** A) Normal follicles showing uniform cellular structures within the granulosa (G) and zona radiata (Zr). The thecal cell layer (solid arrow) and surface epithelium (open arrow) are visible and free of any structural irregularities or apoptosis. B) Follicles of a female zebrafish exposed to 0.5 μg $L^{-1}$ of a carbamazepine, showing separation of the granulosal cell layers (G) from the basement membrane (BM) of the follicle, small cuboidal and irregularly shaped granulosa (rectangulated) and TUNEL positive staining within the thecal cell layer (solid arrow). C) Follicle from a female zebrafish exposed to 0.5 μg $L^{-1}$ of a carbamazepine, showing TUNEL positive staining within the granulosal cell layer (G). D) Follicles from a female zebrafish exposed to 10 μg $L^{-1}$ of a carbamazepine, showing separation of the zona radiate from the granulosa, exposing the basement membrane (BM) and TUNEL positive staining within the thecal cell layer (solid arrow). Tissues were incubated with the apoptotic maker diaminobenzidine (DAB), which appears brown under light microscopy, and then counter-stained with hematoxylin and eosin (60x). Amongst Carbamazepine exposed females, DAB staining was predominately found within the granulosa and zona radiata, indicating that the cell layers which are undergoing rapid cell division are showing signatures of apoptosis leading to an atritic follicle.

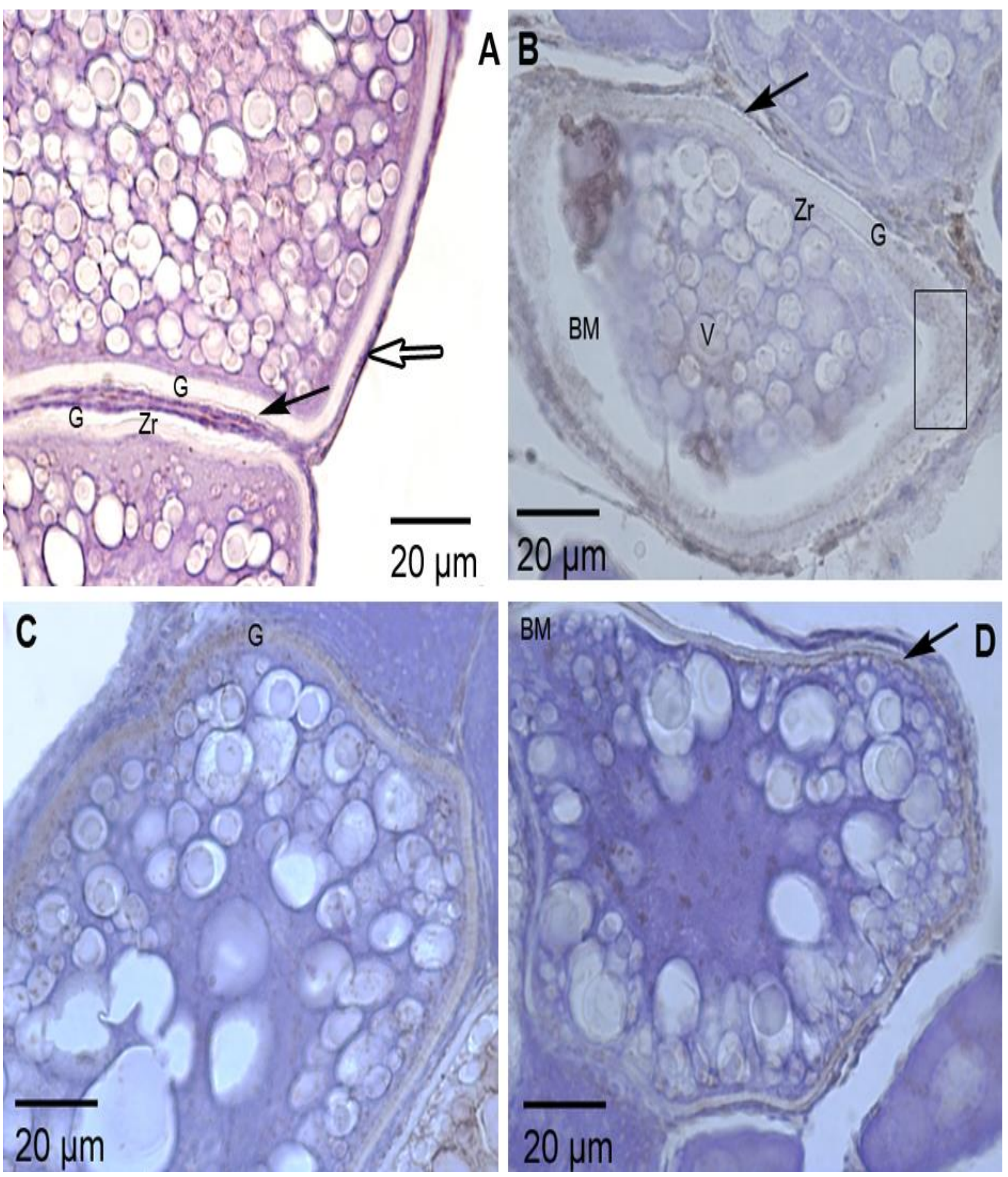
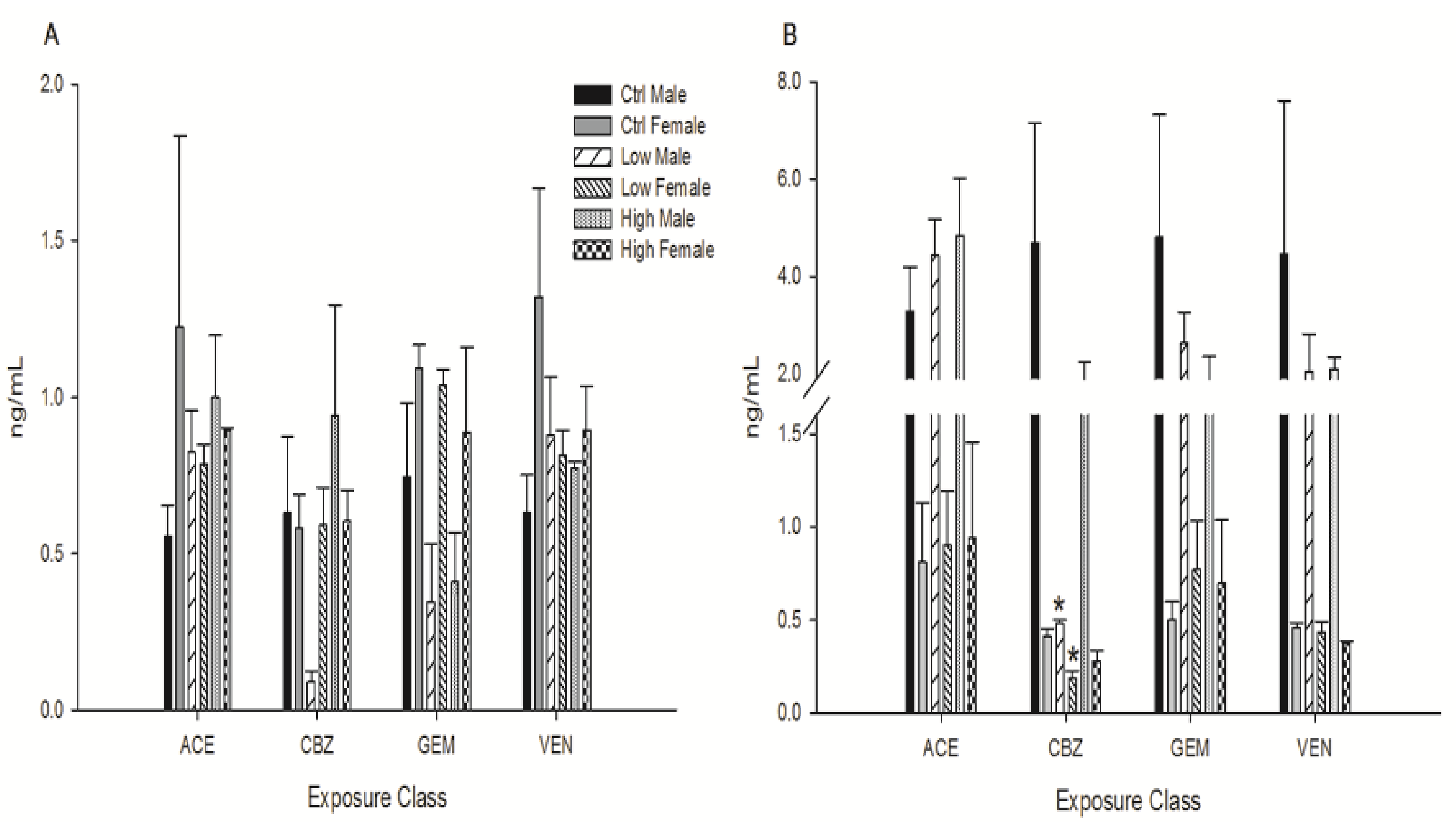

A
G
G
Zr
20 µm
B
Zr
G
BM
V
20 µm
C
G
20 µm
BM
D
20 µm

**Figure 2.7: Plasma concentrations of estradiol and 11-ketotestosterone after pharmaceutical exposure**. Plasma concentrations (ng/mL) of A) Estradiol and B) 11-Ketotestosterone. N=3, * significantly different from controls of the same gender; $p < 0.05$.


A
B
ng/mL
Ctrl Male
Ctrl Female
Low Male
Low Female
High Male
High Female
ACE
CBZ
GEM
VEN
Exposure Class
2.0
1.5
1.0
0.5
0.0
8.0
6.0
4.0
*

## REFERENCES

Andreozzi, R., Raffaele, M., Nicklas, P., 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere 50, 1319-1330.

Beique, J.C., Lavoie, N., de Montigny, C., Debonnel, G., 1998. Affinities of venlafaxine and various reuptake inhibitors for the serotonin and norepinephrine transporters. Eur. J. Pharmacol. 349, 129-132.

Benotti, M.J., Brownawell, B.J., 2007. Distributions of pharmaceuticals in an urban estuary during both dry- and wet-weather conditions. Environ Sci Technol 41, 5795-5802.

Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder, S.A., 2009. Pharmaceuticals and Endocrine Disrupting Compounds in US Drinking Water. Environ Sci Technol 43, 597-603.

Bernet, D., Schmidt, H., Meier, W., Burkhardt-Holm, P., Wahli, T., 1999. Histopathology in fish: proposal for a protocol to assess aquatic pollution. J. Fish Dis 22, 25-34.

Bowley, L.A., Alam, F., Marentette, J.R., Balshine, S., Witson, J.Y., 2010. Characterization of Vitellogenin Gene Expression in Round Goby (Neogobius Melanostomus) Using a Quantitative Polymerase Chain Reaction Assay. Environ Toxicol Chem 29, 2751-2760.

Brooks, S., Tyler, C.R., Sumpter, J.P., 1997. Egg quality in fish: what makes a good egg? Revi Fish Biol Fisheries 7, 387-416.

Brun, G.L., Bernier, M., Losier, R., Doe, K., Jackman, P., Lee, H.B., 2006. Pharmaceutically active compounds in atlantic canadian sewage treatment plant effluents and receiving waters, and potential for environmental effects as measured by acute and chronic aquatic toxicity. Environ Toxicol Chem, 25, 2163-2176.

Cha, Y.I., Kim, S.-H., Solnica-Krezel, L., DuBois, R.N., 2005. Cyclooxygenase-1 signaling is required for vascular tube formation during development. Develop Biol 282, 274-283.

Cha, Y.I., Solnica-Krezel, L., DuBois, R.N., 2006. Fishing for prostanoids: Deciphering the developmental functions of cyclooxygenase-derived prostaglandins. Develop Biol 289, 263-272.

Chang, J.P., Peter, R.E., 1984. Influences of norepinephrine and [alpha]-adrenergic mechanisms on gonadotropin secretion in female goldfish, Carassius auratus. Gen Compar Endocrinol 55, 89-95.

Crockett, E.L., Sidell, B.D., 1993. Peroxisomal beta-oxidation is a significant pathway for catabolism of fatty acids in a marine teleost. Am J Physiol 264, R1004-1009.

David, A., Pancharatna, K., 2009a. Developmental anomalies induced by a non-selective COX inhibitor (ibuprofen) in zebrafish (Danio rerio). Environ Toxicol Pharmacol 27, 390-395.

David, A., Pancharatna, K., 2009b. Effects of acetaminophen (paracetamol) in the embryonic development of zebrafish, Danio rerio. J Appl Toxicol 29, 597-602.

De Lucchini, S., Marracci, S., Nardi, I., 2001. The serotonin 5-HT2B receptor from the puffer fish Tetraodon fluviatilis: cDNA cloning, genomic organization and alternatively spliced variants. Brain Res Mol Brain Res 97, 89-93.

de Oliva, S.U., Miraglia, S.M., 2009. Carbamazepine damage to rat spermatogenesis in different sexual developmental phases. Int J Androl 32, 563-574.

Gervois, P., Torra, I.P., Fruchart, J.C., Staels, B., 2000. Regulation of lipids and lipoprotein metabolism by PPAR activators. Clin Chem Lab Med 38.

Grosser, T., Yusuff, S., Cheskis, E., Pack, M.A., FitzGerald, G.A., 2002. Developmental expression of functional cyclooxygenases in zebrafish. Proc Natl Acad Sci U S A 99, 8418-8423.

Hernandez-Rauda, R., Rozas, G., Rey, P., Otero, J., Aldegunde, M., 1999. Changes in the Pituitary Metabolism of Monoamines (Dopamine, Norepinephrine, and Serotonin) in Female and Male Rainbow Trout (Oncorhynchus mykiss) during Gonadal Recrudescence. Physiol Biochem Zool 72, 352-359.

Hinz, B., Cheremina, O., Brune, K., 2008. Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man. FASEB J. 22, 383-390.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc Natl Acad Sci U S A 104, 8897-8901.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999−2000: A National Reconnaissance. Environ Sci Technol 36, 1202-1211.

Lange, A., Paull, G.C., Coe, T.S., Katsu, Y., Urushitani, H., Iguchi, T., Tyler, C.R., 2009. Sexual Reprogramming and Estrogenic Sensitization in Wild Fish Exposed to Ethinylestradiol. Environ Sci Technol 43, 1219-1225.

Lepage, O., Larson, E.T., Mayer, I., Winberg, S., 2005. Serotonin, but not melatonin, plays a role in shaping dominant-subordinate relationships and aggression in rainbow trout. Horm Behav 48, 233-242.

Lister, A., Regan, C., Van Zwol, J., Van Der Kraak, G., 2009. Inhibition of egg production in zebrafish by fluoxetine and municipal effluents: a mechanistic evaluation. Aquat Toxicol 95, 320-329.

Lister, A.L., Van Der Kraak, G., 2008. An investigation into the role of prostaglandins in zebrafish oocyte maturation and ovulation. Gen Comp Endocrinol 159, 46-57.

Marnett, L.J., Rowlinson, S.W., Goodwin, D.C., Kalgutkar, A.S., Lanzo, C.A., 1999. Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. J Biol Chem 274, 22903-22906.

Meinelt, T., Playle, R.C., Pietrock, M., Burnison, B.K., Wienke, A., Steinberg, C.E., 2001. Interaction of cadmium toxicity in embryos and larvae of zebrafish (Danio rerio) with calcium and humic substances. Aquat Toxicol 54, 205-215.

Mennigen, J.A., Harris, E.A., Chang, J.P., Moon, T.W., Trudeau, V.L., 2009. Fluoxetine affects weight gain and expression of feeding peptides in the female goldfish brain. Regul Pept 155, 99-104.

Mennigen, J.A., Lado, W.E., Zamora, J.M., Duarte-Guterman, P., Langlois, V.S., Metcalfe, C.D., Chang, J.P., Moon, T.W., Trudeau, V.L., 2010. Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, Carassius auratus. Aquat Toxicol 100, 354-364.

Mennigen, J.A., Martyniuk, C.J., Crump, K., Xiong, H., Zhao, E., Popesku, J., Anisman, H., Cossins, A.R., Xia, X., Trudeau, V.L., 2008. Effects of fluoxetine on the reproductive axis of female goldfish (Carassius auratus). Physiol Genomics 35, 273-282.

Mercure, F., Van Der Kraak, G., 1996. Mechanisms of action of free arachidonic acid on ovarian steroid production in the goldfish. Gen Comp Endocrinol 102, 130-140.

Metcalfe, C.D., Miao, X.S., Koenig, B.G., Struger, J., 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ Toxicol Chem 22, 2881-2889.

Miao, X.S., Koenig, B.G., Metcalfe, C.D., 2002a. Analysis of acidic drugs in the effluents of sewage treatment plants using liquid chromatography-electrospray ionization tandem mass spectrometry. J Chromatogr A 952, 139-147.

Miao, X.S., Koenig, B.G., Metcalfe, C.D., 2002b. Analysis of acidic pharmaceutical drugs in the aquatic environment using liquid chromatography–electrospray tandem mass spectrometry. J Chromatogr A 952, 139-147.

Mimeault, C., Woodhouse, A.J., Miao, X.S., Metcalfe, C.D., Moon, T.W., Trudeau, V.L., 2005. The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, Carassius auratus. Aquat Toxicol 73, 44-54.

Nassef, M., Kim, S.G., Seki, M., Kang, I.J., Hano, T., Shimasaki, Y., Oshima, Y., 2010. In ovo nanoinjection of triclosan, diclofenac and carbamazepine affects embryonic development of medaka fish (Oryzias latipes). Chemosphere 79, 966-973.

Perry, S.F., 1997. Proton pumps in the fish gill and kidney. Fish Physiol Biochem 17, 363-369.

Perry, S.F., Shahsavarani, A., Georgalis, T., Bayaa, M., Furimsky, M., Thomas, S.L.Y., 2003. Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: Their role in ionic and acid-base regulation. Journal of Experimental Zoology Part A: Compar Exper Biol 300A, 53-62.

Raldua, D., Andre, M., Babin, P.J., 2008. Clofibrate and gemfibrozil induce an embryonic malabsorption syndrome in zebrafish. Toxicol Appl Pharmacol 228, 301-314.

Ruibal, C., Soengas, J.L., Aldegunde, M., 2002. Brain serotonin and the control of food intake in rainbow trout (Oncorhynchus mykiss): effects of changes in plasma glucose levels. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 188, 479-484.

Runnalls, T.J., Hala, D.N., Sumpter, J.P., 2007. Preliminary studies into the effects of the human pharmaceutical Clofibric acid on sperm parameters in adult Fathead minnow. Aquat Toxicol 84, 111-118.

Seda, M., Pinto, F.M., Wray, S., Cintado, C.G., Noheda, P., Buschmann, H., Candenas, L., 2007. Functional and molecular characterization of voltage-gated sodium channels in uteri from nonpregnant rats. Biol Reprod 77, 855-863.

Sema, I.U., Özlem, C., 2009. Atresia and Apoptosis in Preovulatory Follicles in the Ovary of Danio rerio (Zebrafish). Turk J of Fish Aquat Sci 9, 215-221.

Sorensen, P.W., Hara, T.J., Stacey, N.E., Goetz, F.W., 1988. F prostaglandins function as potent olfactory stimulants that comprise the postovulatory female sex pheromone in goldfish. Biol Reprod 39, 1039-1050.

Terlouw, S.A., Masereeuw, R., Russel, F.G., Miller, D.S., 2001. Nephrotoxicants induce endothelin release and signaling in renal proximal tubules: effect on drug efflux. Mol Pharmacol 59, 1433-1440.

Tixier, C., Singer, H.P., Oellers, S., Muller, S.R., 2003. Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters. Environ Sci Technol 37, 1061-1068.

Triebskorn, R., Casper, H., Scheil, V., Schwaiger, J., 2007. Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (Oncorhynchus mykiss ) and common carp (Cyprinus carpio ). Analy Bioanalytical Chem 387, 1405-1416.

Van den Brandhof, E.J., Montforts, M., 2010. Fish embryo toxicity of carbamazepine, diclofenac and metoprolol. Ecotoxicol. Environ. Saf. 73, 1862-1866.

Weigt, S., Huebler, N., Strecker, R., Braunbeck, T., Broschard, T.H., 2011. Zebrafish (Danio rerio) embryos as a model for testing proteratogens. Toxicol 281, 25-36.

Wood, A.W., Van Der Kraak, G.J., 2001. Apoptosis and ovarian function: novel perspectives from the teleosts. Biol Reprod 64, 264-271.

# CHAPTER 3

# CHRONIC EFFECTS OF EXPOSURE TO A PHARMACEUTICAL MIXTURE AND MUNICIPAL WASTEWATER IN ZEBRAFISH

Reproduced with kind permission from of Aquatic Toxicology.
[Galus, M., Jeyaranjaan, J., Smith, E., Li, H., Metcalfe, C., Wilson, J.Y., 2013.
Aquat Toxicol 132-133, 212-222] © 2013

## ABSTRACT

Pharmaceuticals and personal care products (PPCPs) are discharged in municipal wastewater. Effects in aquatic organisms exposed to individual pharmaceuticals in the laboratory have raised concerns regarding the environmental impacts of PPCPs, yet environmental exposures are always to complex mixtures. In this study, adult zebrafish (*Danio rerio*) showed significantly decreased embryo production after a 6 week exposure to a pharmaceutical mixture (MIX; 0.5 and 10 µg $L^{-1}$) of acetaminophen, carbamazepine, gemfibrozil and venlafaxine and to diluted wastewater effluent (WWE; 5% and 25%). Atretic oocytes and altered ovarian histology were significantly increased in female zebrafish exposed to both concentrations of MIX or WWE, which indicates a direct effect on oocyte development that may account for reduced embryo production. Apoptosis within the thecal and granulosa cell layers was identified in female zebrafish with atresia. Exposures to MIX or WWE at both concentrations severely altered kidney proximal tubule morphology, but no histological impacts on other organs were observed. Exposure of embryos to MIX or WWE at the high concentration significantly increased the incidence of developmental abnormalities. Embryo mortality was elevated with exposure to the high concentration of MIX. These studies indicate that chronic exposure of fish to pharmaceutical mixtures and wastewater impacts reproduction and induces histopathological changes, similar to what we have previously seen with single compound exposures. These data suggest that fish populations exposed to pharmaceuticals discharged in wastewater are at risk of negative impacts to reproductive capacity and health.

## INTRODUCTION

Many studies have documented low concentrations of pharmaceuticals and personal care products (PPCPs) in surface (Sacher et al., 1998), ground (Domagalski et al., 2007), and drinking (Benotti et al., 2008) water. The primary route of entry of PPCPs into the environment is via discharges of wastewater effluents (WWE) from municipal wastewater treatment plants (WWTPs). Pharmaceuticals are present in sewage as a result of the direct consumption and excretion of the parent compound or metabolites in human waste products (Halling-Sorensen et al., 1998), and to a lesser extent, by direct disposal of unused medications (Glassmeyer et al., 2009). Not all pharmaceutical compounds are removed effectively during wastewater treatment (Halling-Sorensen et al., 1998; Kolpin et al., 2002). Some products tend to persist while others are only marginally degraded in the WWTP, or by-pass treatment all together through sewage overflow (Stackelberg et al., 2004). The presence and the concentrations of pharmaceuticals in the aquatic environment are thus determined by prevalence of use, the rates of metabolism in humans, the effectiveness of wastewater treatment, and resistance to degradation in the aquatic environment. Nonetheless, there are strong similarities in North America and Europe in the types and concentrations of pharmaceuticals that have been detected in receiving waters. Concentrations typically are in the ng - μg $L^{-1}$ range (Benotti and Brownawell, 2007; Clara et al., 2005; Lau et al., 2002) and include analgesics, lipid regulators, antibiotics, beta blockers, anti-depressants and steroids (Clara et al., 2005; Kolpin et al., 2002; Lau et al., 2002).

Since pharmaceuticals are designed to induce specific biological responses in humans at low doses and vertebrate taxa share gene homology with high sequence similarity to humans (Gunnarsson et al., 2008; Huggett et al., 2005), laboratory and field based investigations with fish have focused on whether WWE and pharmaceuticals induce responses in teleosts that are analogous to the clinical effects of pharmaceuticals. Although the concentrations of pharmaceuticals found in WWE and receiving waters are much lower than therapeutic doses, chronic exposures and the potential for some pharmaceuticals to bioaccumulate may lead to deleterious effects in aquatic organisms (Cleuvers, 2004). Studies with fish have correlated WWE exposure with elevated stress (Hignite and Azarnoff, 1977), variations in sex steroid levels in both juveniles and adults (Lishman et al., 2006), impaired gonadal development (Sedlak et al., 2000), gonadal intersex (Ings et al., 2011) and decreased reproduction (Lister et al., 2009; Schreck et al., 2001). Laboratory studies have shown that egg production is significantly reduced in sexually mature female zebrafish *(Danio rerio)* exposed to 50% treated wastewater for 7 days (Lister et al., 2009). Intersex and elevated vitellogenin have been documented in wild fish collected at sites downstream from WWE discharges (Brooks et al., 2006; Kavanagh et al., 2004; Vajda et al., 2008; Woodling et al., 2006).

Since natural (17β-estradiol) or synthetic (17α-ethynylestradiol) estrogens are present in WWE, steroids have been the presumed causative agent in many studies that have documented effects on fish reproduction and gonadal development (Tarrant et al., 2008; Vajda et al., 2008). Yet, these effects may not be a consequence of exposure to estrogens alone. Endocrine disrupting chemicals (EDCs) include compounds with multiple

mechanisms of action that can impact a variety of physiological pathways. Several laboratory studies have examined the physiological consequences of exposure to non-estrogenic pharmaceuticals on aquatic vertebrates (Clara et al., 2004; David and Pancharatna, 2009; Mennigen et al., 2008; Mimeault et al., 2006). These studies indicate that multiple pharmaceuticals may have endocrine disrupting capacities that are not likely mediated by steroid receptors.

In our companion study, zebrafish were exposed for 6 weeks to 0.5 and 10 μg $L^{-1}$ acetaminophen (ACE), carbamazepine (CBZ), gemfibrozil (GEM), or venlafaxine (VEN) in single compound exposure experiments (Galus et al., 2013) . The concentrations were chosen based on reported levels in surface water (Andreozzi et al., 2003; Benotti and Brownawell, 2007; Kolpin et al., 2002; Metcalfe et al., 2003; Tixier et al., 2003). The high concentration was near the highest reported concentrations. Since complex PPCP mixtures are found in the environment, many pharmaceuticals are present with similar modes of action and the high concentration is similar to the concentration for all PPCPs within a single drug class. Thus, the concentrations were not meant to be an exact match to what is found at one wastewater treatment plant, or field site, but to represent concentrations that were reasonable and environmentally relevant. Chronic exposure of zebrafish to 10 μg $L^{-1}$ ACE or VEN, and 0.5 and 10 μg $L^{-1}$ CBZ or GEM significantly decreased fecundity (Galus et al., 2013). Atretic oocytes and significantly altered ovarian histology were observed in female zebrafish exposed to CBZ and GEM at both concentrations, indicating a direct effect on oocyte development that may account for reduced reproduction (Galus et al., 2013). Apoptosis within the theca and granulosa cells was identified in exposed female

zebrafish with atretic oocytes by TUNEL positive staining. The incidence of follicular apoptosis was nearly 2 fold higher in exposed females than the controls. Pathological effects on kidney proximal tubules were observed histologically in both male and female zebrafish exposed to all pharmaceuticals at both concentrations. Liver histology was altered by ACE and GEM exposure. Parental exposure to pharmaceuticals did not increase developmental abnormalities, hatching success, or mortality in embryos. However, direct exposures of zebrafish embryos to all pharmaceutical compounds increased mortality and ACE exposure significantly increased developmental abnormalities (Galus et al., 2013). Extrapolation of these results to the environment is difficult as pharmaceuticals are always present as complex mixtures in the environment and only a few studies have characterized the effects of pharmaceutical mixtures (Brian et al., 2006; Cleuvers, 2004; Thorpe et al., 2003). Since mixtures of PPCPs are found within WWE and different classes of pharmaceuticals have varying mechanisms of action (Cleuvers, 2004), it is possible that the effects of single pharmaceuticals cannot be replicated in a mixture of these compounds.

The present study was designed to investigate effects of chronic exposure to low concentrations of a mixture of pharmaceuticals (with different mechanisms of action) and diluted WWE on the reproduction, histopathology, and development of zebrafish. This study determines if a pharmaceutical mixture and a more complex effluent induce similar effects to those seen previously with single PPCP compounds. The compounds used in the pharmaceutical mixture were selected based on prior studies of single PPCP effects in zebrafish (Galus et al., 2013) and were confirmed to be present in wastewater from the WWTP that provided the WWE for exposures. Effects found in single compound, mixture,

and complex effluent exposures would be suitable endpoints for laboratory-based studies of mixture toxicity and drug-drug interactions and field studies downstream of WWE discharge.

## METHODS

### *Wastewater*

All samples of wastewater that were used for a preliminary survey of PPCPs in untreated and treated wastewater and for exposures of zebrafish to WWE were collected from a WWTP located in southern Ontario, Canada. The WWTP serves a population of approximately 480,000 through 2,100 kilometers of sewers in a 40% combined and 60% separated sewer system. The WWTP uses secondary treatment with activated sludge. The average flow rate is 350 ML day$^{-1}$, with a hydraulic retention time of approximately 6.5 hours.

### *Test Chemicals*

Pharmaceutical mixtures (MIX) were composed of equal concentrations of acetaminophen (ACE), carbamazepine (CBZ), gemfibrozil (GEM), and venlafaxine (VEN). For stock solutions, pharmaceutical compounds (Sigma Aldrich, Toronto, ON, Canada) were dissolved in either reverse osmosis treated water (ACE and VEN) or in dimethyl sulfoxide (DMSO; GEM and CBZ) and diluted into 12 L (Experiment I) or 8 L (Experiment II) tanks with system water (distilled water with 12 mg L$^{-1}$ sodium bicarbonate and 60 mg L$^{-1}$ sea salts, Instant Ocean, Spectrum Brands, Madison, Wisconsin USA) for adult exposure experiments. PPCPs were diluted into 48 well plates with E3 media (5 μg L$^{-1}$ NaCl, 0.17 μg L$^{-1}$ KCl, 0.33 μg L$^{-1}$ $CaCl_2$ and 0.33 μg L$^{-1}$ $MgSO_4$) for embryo exposures.

The nominal concentration of each pharmaceutical in the exposure tanks was 0 (control), 0.5 (low) or 10 (high) μg $L^{-1}$. The control and exposure tanks had a final concentration of carrier solvent (DMSO) of 0.004%. A water only control was not used and there were no differences in any measured endpoint (reproductive, developmental, histological, hormonal) across solvent and water controls during experimental exposures with ACE, CBZ, GEM or VEN alone (Galus et al., 2013) . The concentrations of PPCPs in the MIX were based on those used in single compound exposures in Galus et al., (2013).

For the treatments with wastewater effluent (WWE), final treated effluent was collected bi-weekly from the WWTP as a composite 24 hour sample, transported to the lab and stored in the dark at 5°C. WWE was diluted to 5% (low) or 25% (high) with system water or with E3 media for adult and embryo exposures, respectively. Control tanks and wells contained system water or E3 media alone.

*Exposures*

Wild-type, adult zebrafish (DAP International, Canada) were housed at 4 fish $L^{-1}$ and 1:1 sex ratio in a recirculating system (28°C, pH 7-8, dissolved oxygen ≥87% and conductivity 470-455 μS) with ≥10% daily renewal. Aquaria water was made from distilled water supplemented with sodium bicarbonate and sea salts (Instant Ocean, Spectrum Brands, USA) to achieve the conditions above using an automatic dosing system. Fish were fed twice with a commercial food (Tetramin Tropical Flakes, Tetra, USA) and once with live, adult *Artemia* (GSL Brine Shrimp, USA) each day. Zebrafish were kept on a 14:10 hours (light:dark) light cycle. Fish were maintained in our facility for a minimum of one month prior to the onset of the experiment and breeding was initially monitored on a

weekly basis. Fish were sorted to ensure a 1:1 sex ratio, based on a visual external examination prior to the first daily feeding, and reproductive output was determined 3-5 times per week for a minimum of 2 weeks prior to the experiment. Embryo production was compared across tanks and only fish from tanks with equivalent reproductive output were included in the experiment. All animal holding, breeding, and experimentation were performed in accordance to McMaster University's animal care policies and under an approved animal use protocol.

Triplicate tanks for exposure of adult zebrafish were set up with 50 (Experiment I, control, low and high concentration MIX and WWE treatment) or 30 (Experiment II, control and high concentration treatment of MIX only) zebrafish per tank at a sex ratio of 1:1. We did not repeat the WWE exposures for logistical reasons. During Experiment I, all endpoints (reproductive, developmental, hormone, histological) were determined, described below. During Experiment II, only reproductive endpoints were completed to ensure that the effects on reproduction were repeatable and reliable. Tanks were randomly assigned a treatment and moved to exposure tanks 3 days prior to the start of the experiment to acclimate. The mean weight of control, low and high concentration fish, for the MIX exposure, were 403 ± 19, 439 ± 7, and 422 ± 50 mg for males and 439 ± 32, 526 ± 71, and 478 ± 34 mg for females, respectively. The mean weight was not different across treatment groups for either gender. Mean weights were not statistically different across MIX and WWE exposures. All of our treatments were sublethal and mortality was not different across treatments.

The treatments were dosed with test mixtures (MIX) or with WWE diluted in system water for 6 weeks. 6 weeks was chosen to allow a long time course for reproductive effects at low concentration; 2 week exposure of zebrafish significantly decreased reproduction at 50% effluent but not lower concentrations (Lister et al.,2009). Exposure tanks were recirculating systems with a mechanical and sponge filter, thermometer, 25V heater and bio-beads preconditioned with nitrifying bacteria. All tanks were maintained at a temperature of 28 - 29°C and pH of 7.0 - 7.7. Tanks were dosed every three days with a 90% water change, over a period of six weeks. Effluent was diluted directly before water change out and added to the tank in diluted form. Pharmaceutical mixtures were directly dosed, from stock solutions, into each tank. Weekly measurements of pH, dissolved oxygen, conductivity, nitrate, nitrite, and ammonia were conducted.

Fertilized embryos from unexposed parents were collected within 1.5 hours of first light from tanks of brood stock using an embryo trap. Embryos from different tanks were mixed and randomly placed individually in 48 well plates in E3 embryo-rearing media (1 mL well$^{-1}$; 5 µg L$^{-1}$ NaCl, 0.17 µg L$^{-1}$ KCl, 0.33 µg L$^{-1}$ $CaCl_2$ and 0.33 µg L$^{-1}$ $MgSO_4$) containing appropriate concentrations of MIX or WWE. Embryos were incubated at 28.5°C for 72 hours post fertilization (hpf) and examined as described below. At least one 48 well control plate was followed for each embryo collection to determine background mortality in the clutch of embryos. Mortality can be variable across groups of embryo; embryos from unexposed parents typically exhibit less than 10% mortality in our facility. Data from control and treated embryos were excluded when mortality in control plates was

greater than 10%. The number of embryos were N=288 (MIX) and N=336 (WWE) per treatment group.

*Tank Water Samples for PPCP Analyses*

During the third week of MIX exposures with adult zebrafish (Experiment I only), samples of 20 mL of tank water were collected to verify nominal exposure concentrations. Samples were collected from each of the three replicate exposure tanks. Water was sampled at 10 min, 24 hrs, 48 hrs, and 72 hrs after water changes. Water samples were covered in foil and stored at 4°C for no more than 24 hrs prior to extraction. No water samples were taken during embryo exposures.

Twenty-four hour composite samples of final effluent were diluted for the WWE experiment. During the 6 week exposure, 200 ml water samples were collected at 10 min and 24 hr after water changes in each tank. Samples were collected at random across all water changeouts from each of the three replicate exposure tanks to account for the variability in effluent composition throughout the experiment. No water samples were taken during embryo exposures.

A volume of 10 (MIX) or 100 (WWE) ml was prepared for extraction of ACE, CBZ, and GEM by adjusting the pH to 7.5 with 1M NaOH or with 3.5M $H_2SO_4$. An additional 10 (MIX) or 100 (WWE) ml sample was prepared for extraction of VEN by acidifying to pH 3.0 with 3.5M $H_2SO_4$. Samples were spiked with 1 $\mu gL^{-1}$ of stable isotope homologues of ACE, GEM, and CBZ, or VEN prior to extraction so that these surrogate compounds could be used as internal standards for analysis. Extractions were carried out on a Visiprep

solid phase extraction (SPE) manifold (Supelco, Bellefonte, Pennsylvania USA) using Oasis MAX cartridges for ACE, GEM, and CBZ, or Oasis MCX cartridge for VEN. The SPE cartridges were purchased from Waters (Mississauga, ON, Canada). Oasis MAX SPE cartridges were preconditioned by sequentially adding 6 ml MeOH, 0.1 M NaOH, and distilled water. Oasis MCX SPE cartridges were pre-conditioned by sequentially adding 6 mL of acetone, methanol, and HPLC grade water adjusted to pH 2.0 with 3.5M $H_2SO_4$. Water samples were passed through the column at a rate of 5 (MAX) or 10 (MCX) mL $min^{-1}$. Oasis MAX columns were eluted with 2 mL of MeOH and then 3 x 3 mL 2% formic acid in MeOH. Each 3 mL aliquot was allowed to remain in the cartridge for 5 minutes prior to elution. Oasis MCX columns were eluted with 3 x 3 ml of 5% $NH_4OH$ in methanol (v/v), allowing each 3 mL aliquot to remain in the cartridge for 10 minutes prior to elution. All glassware used in these extraction procedures was pre-cleaned with acetone and hexane.

*Wastewater Samples for PPCP Analyses*

In October of 2006 and 2008, 24 hour volume weighted composite samples were collected of untreated wastewater (influent) and treated wastewater (effluent) from the WWTP to determine if the target PPCPs were typically present in the wastewater effluent and to determine a larger number of PPCP analytes. Grab samples of wastewater were also collected from the treatment stream after the primary and secondary clarifiers. The analytical results from these surveys were used to select the target compounds for the mixtures used in laboratory exposures to zebrafish and to compare to our laboratory tank concentrations. The samples were analyzed for the range of PPCPs listed in Supplemental Table S1, including selected base/neutral compounds, acidic pharmaceuticals, beta-blocker

drugs, antidepressants, sulfonamide antimicrobials, antibacterial compounds and EDCs. Wastewater samples were collected in solvent-washed glass bottles, which were rinsed with tap water and HPLC-grade water prior to sampling.

For the initial survey of PPCPs, wastewater samples were prepared for analysis by SPE using methods described by Metcalfe et al. (2010) for antidepressants, Scheurer et al. (2010) for beta-blockers, Topp et al. (2008) for acidic and base/neutral drugs and sulfonamide antibiotics, and Li et al. (2010) for bisphenol A (BPA), estrone, triclosan and triclocarban. All of these compounds were extracted from wastewater samples of 100 mL using SPE with Oasis HLB, MAX or MCX extraction cartridges purchased from Waters (Mississauga, ON, Canada). All samples were extracted in triplicate.

*PPCP Analyses in Extracted Tank Water and Effluent*

The extracts prepared from wastewater were analyzed by high pressure liquid chromatography and tandem mass spectrometry (LC-MS/MS) using methods described by Metcalfe et al. (2010) for antidepressants, Scheurer et al. (2010) for beta-blockers, Topp et al. (2008) for acidic and base/neutral drugs and sulfonamide antibiotics, and Li et al. (2010) for bisphenol A (BPA), estrogen hormones, triclosan and triclocarban. For analysis by LC-MS/MS of the target analytes in MIX or WWE, the VEN extract was analyzed according to methods described by Metcalfe et al. (2010), and the ACE, GEM, and CBZ extract was analyzed according to Li et al. (2010). The ionization source for LC-MS/MS was either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), depending on the target analyte. Stable isotope surrogates for all target analytes were spiked into the water samples as internal standards prior to extraction to aid with sample

quantitation. Concentrations of the target analytes were calculated using an internal standard method with a five-point calibration curve spanning the range of anticipated analyte concentrations in the samples. Limits of quantitation for the target analytes were all within the range of 0.005 - 0.01 μg $L^{-1}$.

*Reproductive and Developmental Endpoints*

During adult exposure experiments (Experiment I and II), embryos were collected daily in a mesh covered plastic tray for 1.5 hours after first light and placed in E3 media at 28.5°C. All eggs were counted and observed at 6 hpf; embryos that were alive and actively dividing were considered viable embryos. Unfertilized eggs were included in counts of total eggs ovulated, those dead at 6 hpf were included in the count of total embryos. Reproductive data was normalized by the numbers of females in the tank, as confirmed by the presence of ovaries at necropsy. During Experiment I, approximately 50 viable embryos $tank^{-1}$ $week^{-1}$ were randomly selected from one day's clutch and observed at 6, 24, 48 and 72 hpf for mortality, hatching and common developmental abnormalities (spinal cord deformations, pericardial edema, yolk sac edema, and stunted growth).

Our egg collection methods use an egg trap placed inside the tank that does not collect every embryo produced. During Experiment II, we modified the design to use 30 fish (but the same density of fish, sex ratio, water, and feeding), with a reduced water column over the egg traps and the recirculating filter shut off during breeding to maximize egg capture. The repeatability and embryo capture rates of our breeding methods, compared to pairwise mating is discussed in detail in Galus et al., (2013) .

*Blood and Tissue Sampling*

After 6 weeks of exposure, adult zebrafish from Experiment I were submerged in ice water and weighed to the nearest mg. An axial incision was made directly below the urogenital opening, severing the caudal artery. A micro-capillary tube was used to collect blood samples from both male and female zebrafish. Blood samples were pooled from 15 - 20 fish (one pool of each gender, per tank) and centrifuged at 21,155 g at 4°C for 12 minutes. The plasma supernatant was collected and stored at -80°C for hormone analysis. Fish were sacrificed by severing the spinal column and gender was confirmed by visual inspection of the gonads; histology samples agreed with assignment of gender at necropsy. The final mean sex ratio between males and females, across all tanks and exposures was 1:0.9. The whole body (open body cavity) was submerged in formalin for a minimum of 48 hr. After fixation, formalin was decanted and zebrafish were rinsed with 50% ethanol for 30 min and stored in 70% ethanol until decalcified with Richard Allen De-cal (Fisher Scientific, Ottawa, ON, Canada) following the manufacturer's protocol. Decalcified samples were transferred to standard histology cassettes and embedded in paraffin.

*Histology*

Histological samples were prepared as previously described (Galus et al., 2013) . Briefly, paraffin embedded whole zebrafish were serially sectioned in the parasaggital plane at 5 µm, deparaffinized with Citrisolv (Fisher Scientific, Ottawa, ON, Canada), rehydrated with 100 – 70% ethanol and stained in hematoxylin and eosin Y (Richard Allen, Kalamazoo, Michigan, USA) following standard histology procedures. Stained slides were examined for histopathological alterations in liver, kidney and gonad on a Zeiss Axiolab

microscope (Carl Zeiss, Hallbergmoos, Germany) following histopathology scores adapted from Bernet el al., (1999). 3-5 sections, approximately 100 – 150 μm apart, were stained and examined individually to identify which sections had the largest cross section for screening each organ (gill, liver, kidney, gonad). At least 2 sections were examined in detail for each organ to determine histopathology scores and rule out staining artifacts and regional differences in staining. The slides were read blind for treatment, sections from the same individual were known, by a single assessor (M. Galus). Histological changes were noted and confirmed by a second assessor (J. Wilson) on a limited set of samples, prior to unblinding the data. The functional units used to identify histopathological changes amongst the liver and kidney and the scoring system to rank the regressive changes were adapted from (Bernet et al., 1999). Oocyte atresia was identified based on significant hyperplasia and hypertrophy amongst the granulosa cells and the degeneration of vitellogenin protein often leading to large gaps between the basement membrane and cell body (Sema and Özlem, 2009).

*TUNEL Assay*

Apoptotic cell death was detected using terminal dideoxynucleotidyl-mediated dUTP nick end labeling (TUNEL), with peroxidase (POD), according to the manufacturer's instructions (Roche Diagnostics, Laval, QC, Canada). Briefly, paraffin sectioned slides had basal levels of peroxidase blocked by incubation with 3% $H_2O_2$ in MeOH (v/v) for 15 min at 23°C. After incubation, slides were rinsed with phoso-buffered saline (PBS) and the TUNEL mixture was placed on the slide and allowed to incubate for 60 min at 37°C. Slides were rinsed with PBS once more and incubated in POD converter solution for 30 min at

37°C before a final rinse with PBS. Slides were then incubated with diaminobenzidine (DAB) (Roche Diagnostics, Laval, QC, Canada) for 60 min and counterstained with hematoxylin and eosin (Richard Allen) before being examined for the presence of apoptosis on a Zeiss Axiolab microscope (Carl Zeiss, Hallbergmoos, Germany). Apoptotic regions appear brown under light microscopy when incubated with the chromogenic substrate DAB. Identification of TUNEL-positive cells was made on the basis of presence or absences of the DAB chromogen. TUNEL staining was completed on samples that had previously been identified as having more severe histological effects (histological scores of 3 or 4 in kidney and 2 in ovary), based on hematoxylin and eosin staining, and control samples. We were unable to complete TUNEL staining on any WWE exposed samples as any remaining tissue, on the histological cassettes, would not yield sections with the desired tissue of interest (kidney in males and females and ovary in females). Testes were present in sections from males stained for the TUNEL assay; thus we have also examined testis for TUNEL positive staining.

*Plasma Hormones*

Plasma samples were extracted and assayed using commercially available enzyme-linked immunosorbent assay kits (Cayman Chemical, Ann Arbor, MI, USA) for the concentrations of estradiol ($E_2$) and 11-keto testosterone (11-KT), as previously described (Bowley et al., 2010).

*Statistical Analysis*

Normality of the data was determined using the Shapiro-Wilk's test. Homogenity of variance was assessed using Levine's test. Cumulative viable embryo production was

normalized by the numbers of females per tank, based on gonadal inspection at dissection. Means and variance were based on triplicate tanks and differences in means were determined using an analysis of co-variance, assessing for embryo production on a per female basis, factoring in concentration and length of exposure. Data was analyzed using SAS statistical software (version 8.02; SAS Institute, Cary, North Carolina USA). The incidence of developmental abnormalities and mortality within larval exposures was analyzed by a one-way analysis of variance (ANOVA). Mortality, hatching, and developmental abnormalities were normalized to the amount of viable or already hatched embryos at 72hpf. Histological data was analyzed based on the percent incidence of histopathology and mean pathological score, within each exposure. Differences in percent incidence and mean score were analyzed using a student's *t* test. Plasma hormone concentrations did not pass normality and therefore were analyzed using a Kruskal–Wallis one-way analysis of variance on ranks. Statistical analyses on adults were completed within a single gender, comparing responses in control to exposed fish from males and females separately. All statistical tests, except that involving cumulative viable embryo production were analyzed using SigmaPlot 11.0 (Systat Software). Following ANOVA analysis, significant differences were determined using a Holm-Sidak post-hoc test. All data was expressed as mean ± standard error. The significance level for all statistical analyses was set at $P$=0.05.

## RESULTS

*PPCPs in Wastewater*

Samples of untreated and treated wastewater from the WWTP were analyzed in triplicate for a range of PPCPs to ensure the PPCPs used in the single compound and MIX exposures were present in the effluent stream from the WWTP providing effluent, as well as to compare to our tank concentrations during the WWE exposures. A more extensive range of PPCPs was analyzed in the samples collected in 2008 than in the samples collected in 2006 (Supplemental Table S5). Pharmaceuticals that were analyzed in both sampling years were found at approximately the same concentrations. Rates of removal were determined by comparing the concentrations in the final treated wastewater (effluent) from the concentrations in the untreated wastewater (influent). For both sampling periods, most of the PPCPs showed similar rates of removal, with the exception of GEM and trimethoprim. The removal rate for GEM was 89% and 46% for 2006 and 2008, respectively. The removal rate for trimethoprim was 21% and 40% for 2006 and 2008, respectively. Some pharmaceuticals (CBZ, VEN, fluoxetine) had low removal rates (<20%). For instance, CBZ was not removed by treatment in either year. High rates of removal (<80%) were found for most of the acidic compounds (Supplemental Table S5). Interestingly, fluoxetine in 2006 and propranolol in 2008 were found in higher concentrations in the effluent than in the influent. Since the composite samples of treated and untreated wastewater were collected at the same time and the hydraulic retention time in the WWTP is approximately 6-7 hours, these data may reflect temporal variations in the concentrations of target analytes entering the WWTP in the wastewater.

*Measured Concentrations*

Analyses were conducted to determine the measured pharmaceutical concentrations within the aquaria. Since tank water was renewed every third day, individual water samples were taken at 10 min and 24, 48 and 72 hours after dosing from each replicate tank. All of these samples were analyzed in the MIX treatments, but only samples from the 10 min and 24 hr time points were analyzed for the WWE treatments (Supplemental Table S6). The concentrations of ACE were lower in the treatments at 72 hours post water change relative to the starting concentrations. For the other target analyates, the concentrations remained relatively stable over the first 48 hours, with concentrations declining at 72 hours after renewal. In several samples collected at 10 min, the target analytes were not detected, perhaps reflecting incomplete mixing in the tank immediately after dosing (Supplemental Table S6).

In the MIX exposure, the mean aqueous concentrations of CBZ and GEM were close to the nominal concentrations in treatments for both the low and high concentrations. For ACE and VEN, the concentrations in the high concentration treatments were approximately 80% and 50% lower than the nominal, respectively. ACE was not detected at concentrations greater than the limits of quantitation (LOQ) in the low concentration treatment at 10 min post-dosing, but at the 24 hr time period the mean concentration was $0.09 \pm 0.16$ μg $L^{-1}$, approximately 80% below the nominal concentration. The low VEN concentration was approximately 90% of the nominal concentration (Supplemental Table S6).

Pharmaceutical concentrations during the WWE exposure were significantly lower than the concentrations in the MIX exposure (Supplemental Table S6). GEM concentrations were on average approximately ≥60% higher than any other pharmaceutical, yet the low and high exposure tanks had similar concentrations (Supplemental Table S6). Concentrations of ACE and VEN were below LOQs in both control and WWE low concentration tanks, but CBZ and GEM were present at concentrations >LOQ in WWE treatments at both the low and high concentrations. All pharmaceuticals present at detectable concentrations were at levels less than 0.3 μg $L^{-1}$, even in the high (25% WWE) treatment tanks.

Limits of quantitation for the target analytes were all within the range of 0.005 - 0.01 μg $L^{-1}$. While ACE was below LOQ for all time points in the control tanks, CBZ, GEM, and VEN were above LOQ in the control tanks of the MIX experiment for some time points. Yet, concentrations in low concentration tanks were typically double measured concentrations in control tanks except for VEN. Concentrations of VEN were similar between the control and low concentration tank. For the WWE exposure, the control tanks were below LOQ for all analytes at both time points.

*Embryo production*

In experiments with adult zebrafish exposed to MIX or WWE for 6 weeks, there was a significant reduction in the average cumulative number of viable embryos produced per female, relative to the control treatments (Figure 3.1). However, there were no statistically significant differences in embryo production between low and high concentrations of MIX (Figure 3.1A) or WWE (Figure 3.1B). Reproductive impacts were

repeatable. Experimental exposures were repeated for control and high concentration of MIX with 30 fish per tank (see methods for details). Embryo production was reduced significantly from controls with exposure to high concentration MIX (70% in Experiment I versus 60% in Experiment II).

*Effects on Embryos*

There was no effect on the survival, hatching success or incidence of developmental abnormalities (data not shown) in embryos produced by adult zebrafish exposed to MIX or WWE at either concentration. Direct exposure of embryos collected from unexposed parents to 10 μg $L^{-1}$ MIX caused a significant increase in mortalities (25% mortality) relative to controls (4% mortality, Figure 3.2A). Surprisingly, WWE exposure caused a significant decrease in embryo mortality: mortalities in embryos exposed to 5% and 25% dilutions of WWE were lower by 33 and 23 %, respectively, than mortalities in the control treatment (Figure 3.2A).

Zebrafish embryos exposed to 10 μg $L^{-1}$ MIX and 25% WWE showed a 24 and 1.8 fold increase in developmental abnormalities, respectively, when compared to controls (Figure 3.2B). This increase was primarily caused by an increase in the occurrence of yolk-sac edema (data not shown). Interestingly, multiple abnormalities (yolk-sac edema and spinal deformations) were observed in 1.1 and 3.1% of zebrafish embryos exposed to 0.5 and μg $L^{-1}$ of MIX, respectively but not in any of the control or WWE exposed animals. Hatching success was not affected by embryonic exposure to any MIX or WWE treatments and >99% of all embryos were hatched by 72 hpf.

*Histology and TUNEL Staining*

Exposure of adult zebrafish to MIX or WWE at all concentrations resulted in a significant increase in the mean histopathological score and incidence of regressive changes within the kidney tubule (Supplemental Table S7). At least 74% of the exposed animals showed signs of regressive changes in the proximal tubules of the kidney and the incidence was similar across test concentrations and between males and females. The severity of regressive changes in kidney proximal tubules increased significantly between the low and high concentrations of MIX or WWE. Several male and female fish from both treatments showed the most severe histopathological damage (i.e. index score of 4). Almost all sections examined from exposed animals displayed some form of regressive changes to the proximal tubules, ranging from very minor (Figure 3.3B) to extreme (Figure 3.3D).

Exposure to 0.5 and 10 μg $L^{-1}$ MIX and 5% and 25% WWE caused a significant increase in the occurrence of atretic oocytes in female zebrafish (Supplemental Table S8). Animals from both exposures primarily had multiple atretic oocytes with only a few oocytes that showed normal signs of development (Figure 3.4B). In adult female zebrafish exposed to MIX, the incidence of apoptotic ovarian follicles was 1.5 fold higher in exposed females when compared to controls. Ovarian follicles were judged to be apoptotic based on the criteria of TUNEL positive staining within the theca and granulosa, as well as morphological changes within these layers (Figure 3.5B) (Sema and Özlem, 2009; Wood and Van Der Kraak, 2001). However, the TUNEL positive cells were only limited to early vitellogenic oocytes (S1) and strictly contained within the somatic tissue in controls. There was no presence of any TUNEL positive staining in mature (S3/S4) follicles or within the

theca and granulosa of all control sections (Figure 3.5A) that showed S1 TUNEL positive cells. Females exposed to MIX which stained TUNEL positive, showed hypertrophic cells within the granulosa and vacuolization of the zona radiata. Separation of the granulosa from the basement membrane of the follicle was observed in 60% of MIX exposed females (Figure 3.5B). TUNEL positive ooplasmic vesicles were only observed in exposed females with ovarian apoptosis.

In fish exposed to MIX or WWE, at both the low and high concentrations, there were no histological alterations observed in hepatic or testis morphology. All developmental stages of spermatogenesis, including spermatozoa, were observed in the testis of all males.

*Sex Steroid Concentrations*

For both male and female zebrafish, mean plasma estradiol concentrations were similar amongst treatments (Supplemental Figure S1). Plasma 11-ketotestosterone levels amongst males from the WWE exposure showed larger variations in mean concentration compared to control animals. There was a trend of lower levels of 11-ketotestosterone in males exposed to 25% effluent relative to control males that approached significance ($p=0.061$).

## DISCUSSION

Zebrafish were exposed chronically to low concentrations of a mixture of four PPCPs (ACE, CBZ, GEM and VEN) or to diluted effluent collected from a wastewater treatment plant with secondary treatment using activated sludge. We have previously

exposed zebrafish to these PPCPs individually, at the same concentrations, to determine if the individual compounds had effects on adult or embryonic fish (Galus et al., 2013) . The PPCP concentrations were chosen based on reported levels in surface water (Andreozzi et al., 2003; Benotti and Brownawell, 2007; Brun et al., 2006; Kolpin et al., 2002; Metcalfe et al., 2003; Tixier et al., 2003). The high concentration was near the highest reported concentrations. Water flow rate at a given site will be critical for determining the exact dilution of WWE after discharge, as flow conditions are critical for determining surface water contamination (Kolpin et al., 2004). Yet, there is limited data to calculate the dilution of effluent in receiving waters. In the Höje River, concentrations ranged from 0.9-75% effluent over the year but concentrations were >3.5% or >7% effluent for 9 or 5 months, respectively (Bendz et al., 2005). Wastewater effluent represented 40-100% of the flow for the Thames River, Ontario (Lishman et al., 2006). The concentrations used in this, and our prior study (Galus et al., 2013) , were not meant to be an exact match to what is found at one wastewater treatment plant, or field site, but to represent concentrations that are reasonable and environmentally relevant.

The results from this study show that adult zebrafish exposed to mixtures of ACE, CBZ, GEM and VEN at 0.5 and 10 μg $L^{-1}$ and to dilutions of treated wastewater (WWE) showed a significant decline in embryo production (i.e. viable embryos normalized by number of females per tank), elevated oocyte atresia in the ovaries of females, and histological alterations to the kidneys. Exposure of zebrafish embryos to the mixtures caused reduced survival and induced various developmental alterations, particularly pericardial edema. These results with pharmaceutical mixtures and with WWE support our

earlier observations of sublethal effects in zebrafish exposed to these same pharmaceuticals individually (Galus et al., 2013) . Exposure to ACE, CBZ, GEM and VEN, individually, all caused a decline in embryo production, induced regressive changes within the kidney proximal tubule and increased embryo mortalities. It is interesting that all of these compounds, either individually (Galus et al., 2013) or as a mixture (this study) caused similar responses in zebrafish, considering that ACE is a cyclooxygenase inhibitor, CBZ inactivates voltage gated sodium channels, GEM is a peroxisomal proliferator-activated receptor alpha (PPARα) ligand, and VEN is a selective serotonin and norepinephrine reuptake inhibitor. The mechanisms of action and potential means by which these compounds may cause their effects are discussed in detail in our companion paper (Galus et al., 2013) .

The target pharmaceuticals selected for testing in the MIX treatments were all detected in treated wastewater in the surveys of the WWTP conducted in 2006 and 2008 (Supplemental Table S5). These compounds represent several chemical classes with different modes of action for human therapy. ACE, but not CBZ and VEN, was removed efficiently during wastewater treatment. The concentrations of the target compounds detected in the WWTP survey were similar to the measured concentrations in the high concentration treatment (24 hr) with WWE. For example, ACE was detected in this treatment at a concentration at 0.03 μg $L^{-1}$ and the final effluent concentration within the WWTP in 2006 was 0.05 μg $L^{-1}$. ACE was present in wastewater at higher concentrations than the other acidic drugs surveyed in 2006 but not 2008, and the concentrations of VEN in wastewater were much higher than the other antidepressants surveyed (i.e. citalopram,

fluoxetine, sertraline) at least in 2008. The concentrations of pharmaceuticals reported for the WWTP are similar to previously published studies on the concentrations of pharmaceuticals in the wastewater of Canadian WWTPs (Kolpin et al., 2002; Metcalfe et al., 2003).

*Reproductive Endpoints*

In adult zebrafish exposed to both concentrations of the pharmaceutical mixture and WWE, there was a significant decline in the production of both total (data not shown) and viable embryos. This effect was repeatable and the percentage reduction in embryo production in high concentration MIX compared to control was similar in Experiment I and II. The major differences between Experiment I and II was that the number of fish per tank was less and the embryo capture rate was larger (see materials and methods (Galus et al., 2013) for details). Our results for reduced embryo production in exposed fish are consistent with other studies that showed reproductive impacts in fish exposed to pharmaceuticals (Clara et al., 2004; David and Pancharatna, 2009; Galus et al., 2013; Lister et al., 2009; Mennigen et al., 2008; Parrott and Blunt, 2005), and to wastewater (Ings et al., 2011). However, to the best of our knowledge, the present study is the first to demonstrate that municipal effluent, from a developed country with a predominantly post-industrial economy, diluted to 5% can impact embryo production. Zebrafish exposed to 1 and 10% municipal effluent did not induce effects on reproduction in a previous study (Lister et al., 2009), although we found statistically significant differences in fecundity with WWE exposure by 2 weeks (the same time course used in the Lister study).

The Lister study (2009) raised concerns that the total ammonia in their 50% effluent treatment was so high that it may have exacerbated the reproductive effects observed in that experiment. In the present study, ammonia concentrations were measured throughout the exposure period and in the tanks dosed with effluent, ammonia concentrations always ranged from 0.8 – 1.5 mg $L^{-1}$, which are well below the lethal threshold, but may have affected the fish sublethally. Clearly, wastewater is a complex mixture with variation in composition with time (e.g. 2006 versus 2008 samplings of the same wastewater treatment plant, Supplemental table S5) and with treatment processes. Yet, reproductive effects appear to be a consistent outcome of WWE exposure to fish in laboratory experiments. Considering that multiple classes of pharmaceuticals impact fish reproduction (Galus et al., 2013) , distinguishing whether pharmaceutical concentrations and/or other components (e.g. ammonia as raised by Lister et al., (2009)) are driving the declining reproduction with WWE exposure will be important. Certainly, reproduction is likely a very good endpoint for determining pharmaceutical interactions in fish. For environmentally relevant concentrations, exposure will likely need to be on the order of at least 2-4 weeks.

*Embryonic Exposures*

Direct exposure of embryos to 10 μg $L^{-1}$ MIX resulted in elevated mortalities and developmental abnormalities. Significantly, exposure of embryos to ACE, CBZ, GEM and VEN individually significantly increased embryo mortalities, and exposure to ACE alone increased developmental abnormalities, in particular spinal deformations and yolk and pericardial edema (Galus et al., 2013). The 0.5 μg $L^{-1}$ MIX exposure did not significantly impact mortality, yet exposure of embryos individually to 0.5 μg $L^{-1}$ ACE, CBZ, GEM or

VEN increased embryonic mortality (Galus et al., 2013), suggesting pharmaceutical interactions and non-additive mixture toxicity that will require careful study.

It is interesting that the effects on embryos are only seen with direct exposure and not with parental exposure alone. This suggests that while the fecundity of the parents are impacted, those embryos that are produced are healthy and normal and only developmental exposure will impact survival and abnormalities. In conflict with our MIX exposures, embryos exposed to 5% and 25% WWE showed reduced mortality (Figure 3.2A), indicating that the lower concentrations of pharmaceuticals present in these treatments did not have a negative effect on embryo survival and some component of the WWE was beneficial for survival. Predicting the developmental effects of pharmaceutical mixtures and WWE will be difficult and will require attention to potential pharmaceutical interactions, WWE composition, and developmental impacts of non-pharmaceutical components of WWE.

*Histology*

Exposure of zebrafish to both MIX or WWE, at all concentrations, resulted in regressive changes to the kidney proximal tubules, an effect found in our earlier studies with zebrafish exposed individually to ACE, CBZ, GEM and VEN at both 0.5 and 10 μg $L^{-1}$ (Galus et al., 2013). This effect is a consistent sublethal effect of exposure to pharmaceuticals, regardless of the therapeutic mode of action of the drug or whether fish are exposed to single compounds or mixtures. Since the teleost kidney is primarily involved in the excretion of exogenous solutes, any damage to its functionality could result in a decrease in capacity for excretion and compromise other organ systems (Perry et al.,

2003). Further testing of the physiological impacts of pharmaceutical induced kidney tubule regression is needed to understand whether osmoregulation, pH balance or xenobiotic excretion are impacted by exposure to pharmaceuticals.

CBZ and GEM at 0.5 and 10 μg $L^{-1}$, but not ACE and VEN, caused oocyte atresia in zebrafish (Galus et al., 2013). Consistent with that finding, ovarian damage and particularly the presence of atretic oocytes was observed in adult zebrafish exposed to MIX (0.5 and 10 μg $L^{-1}$) and WWE (5 and 25%), suggesting a consistent sublethal effect of at least some pharmaceuticals and pharmaceutical mixtures on ovaries and the probable cause of the reproductive decline in exposed fish. With MIX or WWE exposures in the present study, the proportion of exposed females with oocyte atresia approached 60%. Oocyte atresia is an uncommon event in healthy female fish and atresia was not observed in any of the control females from this study. Oocyte atresia has been primarily linked to starvation and prolonged exposure to an environmental stressor (Brooks et al., 1997; Wallace and Selman, 1981). Given that our animals were fed to satiation three times a day, nutritional deprivation was likely not the cause of the atresia and exposure to the test chemicals was the causative agent.

*Mixture Toxicity*

There are three common models of mixture toxicity: concentration addition, subtraction, and synergy (Cleuvers, 2003, 2004). Concentration addition predicts that the combined effect of pharmaceuticals will be the sum of the effects observed with each pharmaceutical alone. For compounds with different mechanisms of action, additive effects may not be expected and the combined effects may be less (subtractive) or more

(synergistic) than the sum of the effects by each compound alone (Cleuvers, 2003). In developing mouse embryos, exposure to CBZ in combination with the anti-inflammatory drug ACE reduced embryo survival and increased alterations to gastrulation and segmentation, compared to CBZ exposure alone (Robert, 1999). This synergistic effect was mediated by a drug interaction, as CBZ increased the absorption of ACE (Robert, 1999). Pharmaceutical interactions in mammals are commonly mediated by alterations in pharmacokinetics, particularly via altered hepatic metabolism.

While our purpose was not to explicitly test mixture models, the compounds examined have very different biological mechanisms of action in mammals and the data indicate that the additive model is not applicable to some endpoints measured. Indeed, our data argue for detailed mixture toxicity experiments with pharmaceuticals and other components of WWE in fish. In the MIX exposure, the decline in embryo production (74% at 0.5 μg $L^{-1}$) was less than expected, based on reductions in embryo production that we observed previously in individual drug exposures with ACE (20%), CBZ (34%), GEM (40%) and VEN (0%) (Galus et al., 2013) . Kidney tubule regression was found in 96% and 85% of animals exposed at the low concentration of MIX or WWE. The incidence of kidney damage that we observed previously (Galus et al., 2013) in zebrafish exposed individually to ACE (80%), CBZ (89%), GEM (65%) and VEN (74%) was similar to that observed with the mixtures. However, the severity of the regressive changes was increased in MIX or WWE exposed animals and nearly 90-95% of animals had a severity score of 3 or 4. In all single compound exposures, only 25-30% of animals had a severity score 3 or 4 for kidney tubule regression (Galus et al., 2013). Lastly, liver histopathology was

impacted by ACE and GEM alone and plasma 11-KT levels were impacted by CBZ alone (Galus et al., 2013) but these effects were not seen with the pharmaceutical mixture or with the WWE exposed animals, suggesting modified effects with mixture exposure.

Regardless of whether the effects observed were additive, most of the effects of single pharmaceutical exposure were seen in both MIX or WWE exposures and novel effects were not obvious (e.g. histological impacts in novel organs). Studies of mixture toxicity are quite difficult; even more so under chronic exposures such as used in this study. Differences in reproduction across treatments were statistically significant after 2 weeks, suggesting that exposures from 2-6 weeks would be appropriate to study reproductive toxicity of mixtures. Since all single compounds (Galus et al., 2013), MIX or WWE exposures caused embryonic effects with direct exposures, detailed mixture toxicity tests with embryonic endpoints are warranted and can be more practically completed in zebrafish where exposures are less than one week.

The responses in adult and embryo zebrafish to pharmaceutical mixtures or WWE indicate that there are consistent multi-organ impacts in fish exposed to pharmaceuticals. Of course, in the WWE exposures, it cannot be ruled out that the responses are due to exposure to other components of wastewater and are not induced by pharmaceuticals. However, the present study and our previous study indicate that the same effects on embryo production, kidney pathology, oocyte atresia and developmental abnormalities in embryos are observed when zebrafish are exposed to pharmaceuticals individually or in mixtures, or to diluted wastewater. Given the consistent effects seen with single pharmaceuticals and a pharmaceutical mixture, there is a high probability that the pharmaceuticals detected in

WWE are the causative agents for the endpoints observed. Our data suggests that fish populations may be at risk of adverse effects from chronic exposure to the low concentrations of pharmaceuticals in surface waters that originate from wastewater treatment plants that use secondary treatment with activated sludge.

## ACKNOWLEDGEMENTS

The authors thank the members of the Wilson laboratory, in particular Lucas Bowley, James Harskamp, Nina Kirischian, Patrick Schenck, John Soleas, Marcus Scornaienchi, Shawn Parascandalo and Mika Yoshikawa for sampling assistance. Funding was provided by the Natural Sciences and Engineering Research Council of Canada in the form of a Strategic Project Grant to JYW and CDM (No. 90340311), with the City of Hamilton, Water and Wastewater Division, as a partner agency. The Canada Foundation for Innovation and the Ontario Innovation Trust provided infrastructure support to JYW and to CDM. This research was conducted in accordance with the accepted standards of humane animal care and approved under the McMaster University Animal Research Ethics Board.

**Figure 3.1: Cumulative daily embryo production from zebrafish in tests with breeding fish chronically exposed to a pharmaceutical mixture (A) and wastewater effluent (B).** Mean embryo production is shown as the cumulative mean viable embryo production per female from triplicate tanks. Fish were exposed for 6 weeks to 0 (control), 0.5 (low) and 10 (high) $\mu g\ L^{-1}$ each of a mix of acetaminophen, carbamazepine, gemfibrozil and venlafaxine (A) and 0 (control), 5% (low) and 25% (high) wastewater effluent (B). The carrier solvent, DMSO was dosed at 0.004% in control tanks for the pharmaceutical mixture exposure. * Indicates significant difference from controls, $p \leq 0.05$.

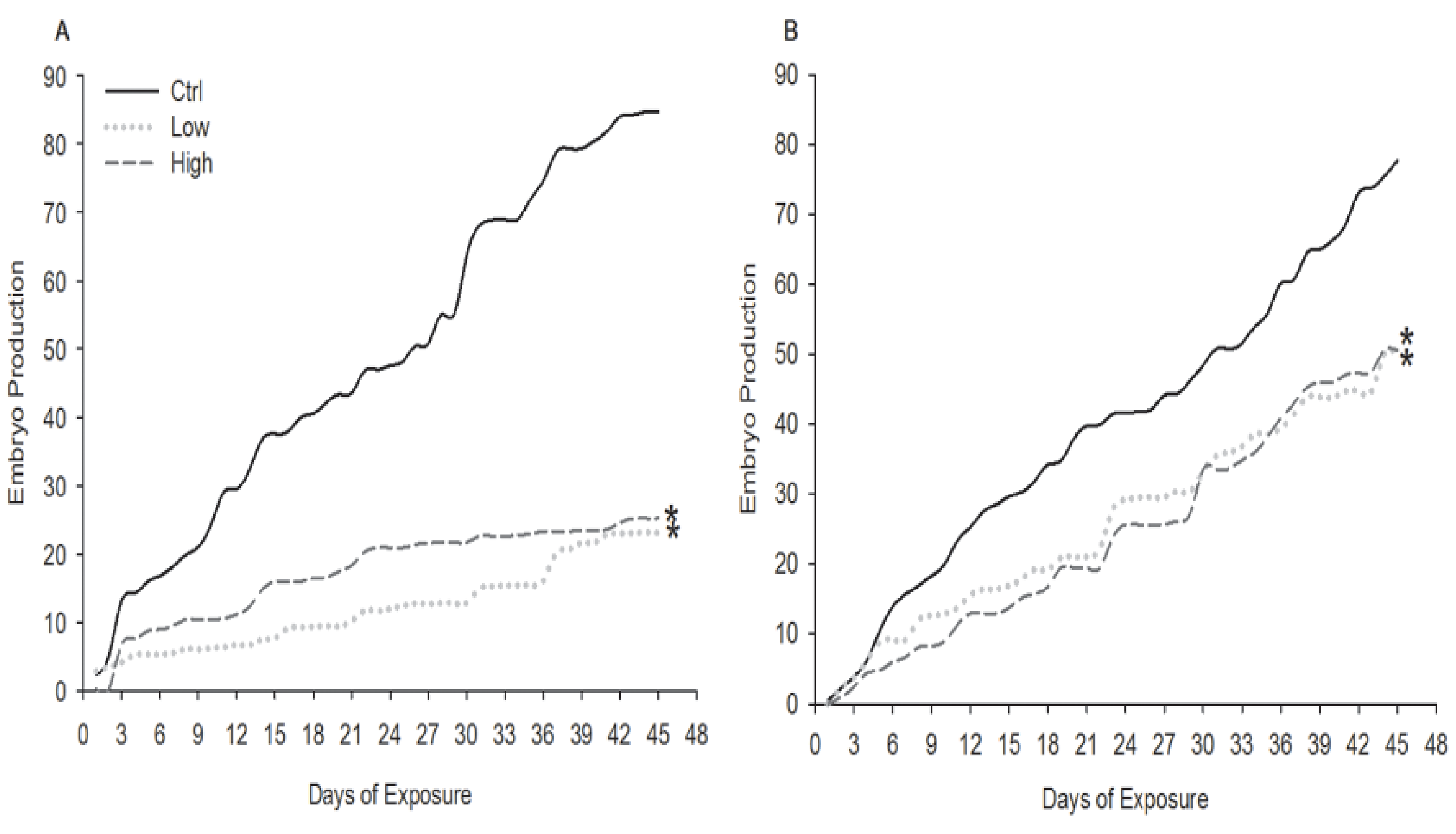

A
B
Ctrl
Low
High
Embryo Production
Days of Exposure
0
10
20
30
40
50
60
70
80
90
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48
**

**Figure 3.2: Mean (N=3) percent total mortalities (A) and developmental abnormalities (B) in zebrafish embryos after exposure to a pharmaceutical mixture (MIX) and to wastewater effluent (WWE).** Embryos from unexposed parents were exposed immediately after collection (≤1.5 hpf) to 0 (control), a mixture of 0.5 (low) and 10 (high) $\mu g\ L^{-1}$ each of acetaminophen, carbamazepine, gemfibrozil and venlafaxine (MIX) and 0 (control), 5% (low) and 25% (high) wastewater effluent (WWE) for 6 weeks. The carrier solvent, DMSO was added at 0.004% to the control tanks for the MIX exposure. Embryo numbers were N=288 (MIX) and N=336 (WWE). Bars which are denoted by different letters were statistically different from each other. Significance is within each exposure and not across exposures $p \leq 0.05$.

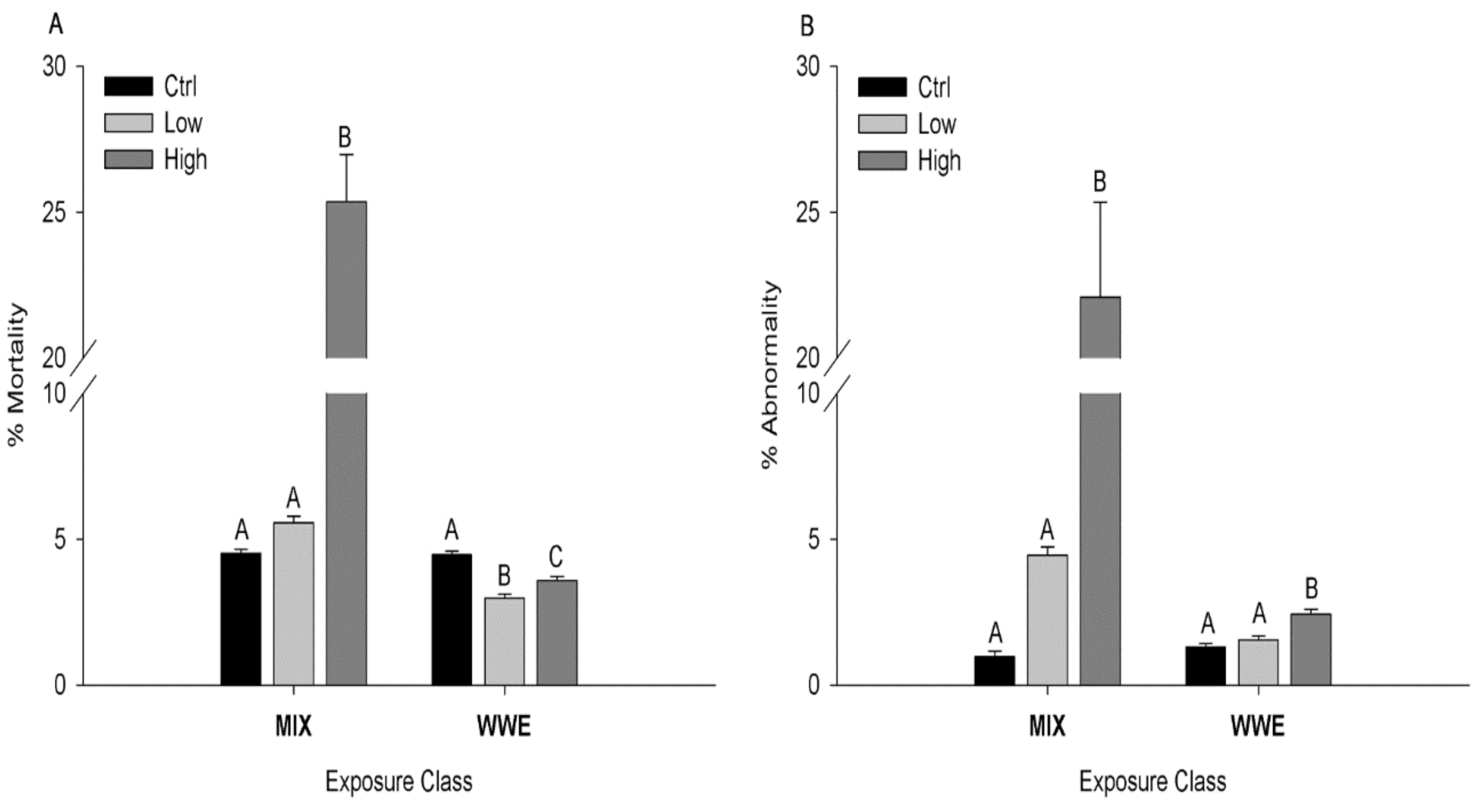

A
Ctrl
Low
High
% Mortality
30
25
20
10
5
0
A
A
B
A
B
C
MIX
WWE
Exposure Class
B
Ctrl
Low
High
% Abnormality
30
25
20
10
5
0
A
A
B
A
A
B
MIX
WWE
Exposure Class

**Figure 3.3: Histology of H&E stained sections (60 x) of the kidney from adult zebrafish from control and exposed treatments.** A) Normal kidney tubules in a male zebrafish from the control treatment showing central nuclei positioning within a completely stained cytoplasm and uniform nuclei within a kidney tubule bundle (arrows). B) Kidney tubules from a male zebrafish exposed to high concentration of MIX showing cells lacking nuclei (arrow), early stages of cytoplasmic degradation (arrow head) and variations in nuclei size (open arrow head); Index score of 2. C) Kidney tubules from a female zebrafish exposed to 25% WWE showing loss of cytoplasmic staining integrity (arrow), nuclei vacuolization and variation in nuclei size (arrow head), and complete loss of cytoplasmic staining with non-central nuclei positioning (open arrow head); Index score of 3. D) Kidney tubules of a male zebrafish exposed to 25% of WWE showing severe loss of cytoplasmic staining integrity (arrow), sickle shaped nuclei (arrow head), and complete loss of cytoplasmic staining with non-central nuclei positioning (open arrow head); Index score of 4.

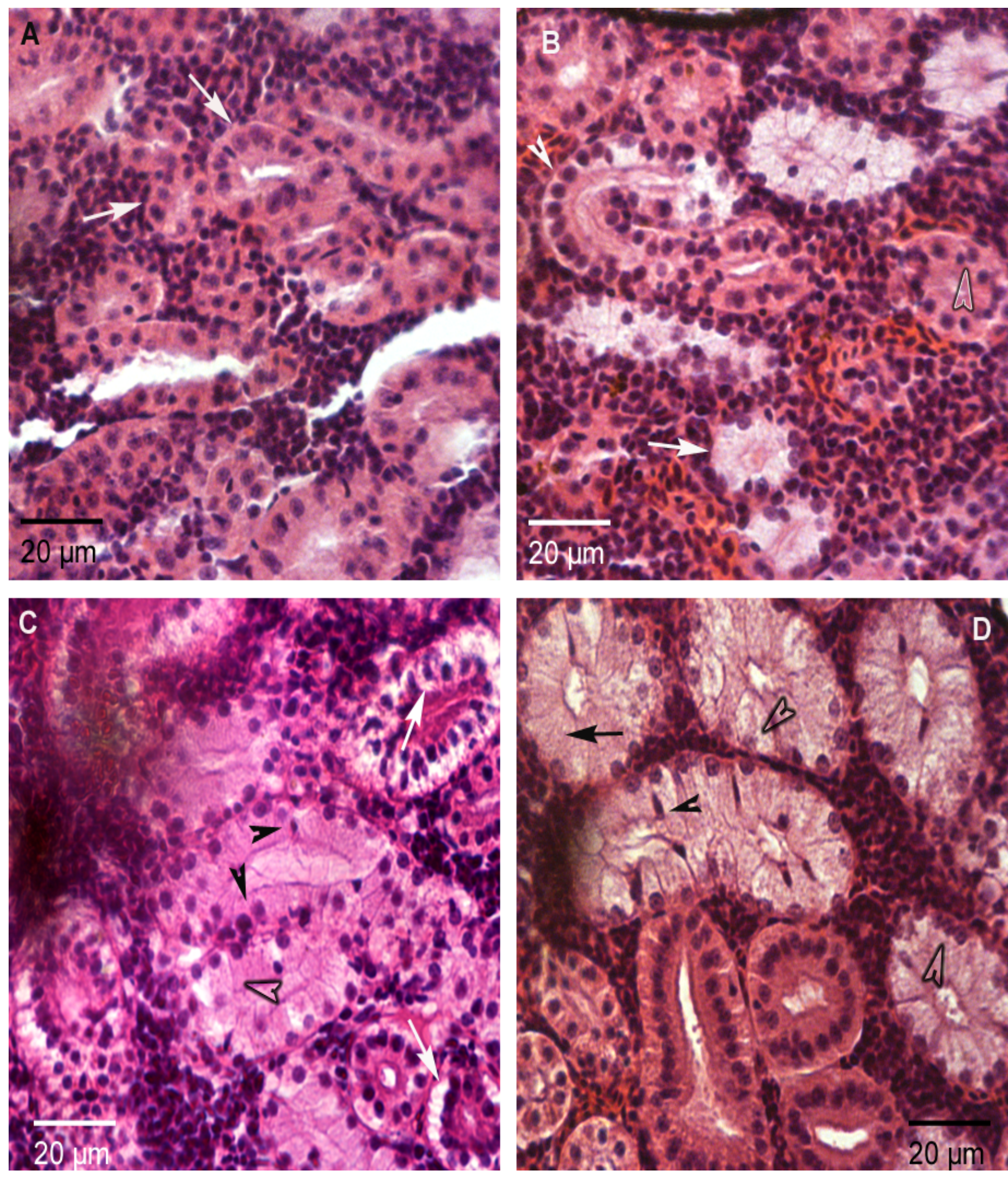

A
20 µm
B
20 µm
C
20 µm
D
20 µm

**Figure 3.4: Histology of H&E stained sections (20 x) of the ovaries from adult female zebrafish from the control treatment and the treatment with exposure to wastewater effluent (WWE).** A) Normal ovary from a control female at the intermediate vitellogenic stages of development. Pre-vitellogenic oocytes (I and II) are mainly located within the periphery, whereas vitellogenic oocytes (III and IV) are located centrally within the ovarian tissue. B) Ovary of a female zebrafish exposed to 5% WWE. Arrows identify atretic oocytes.

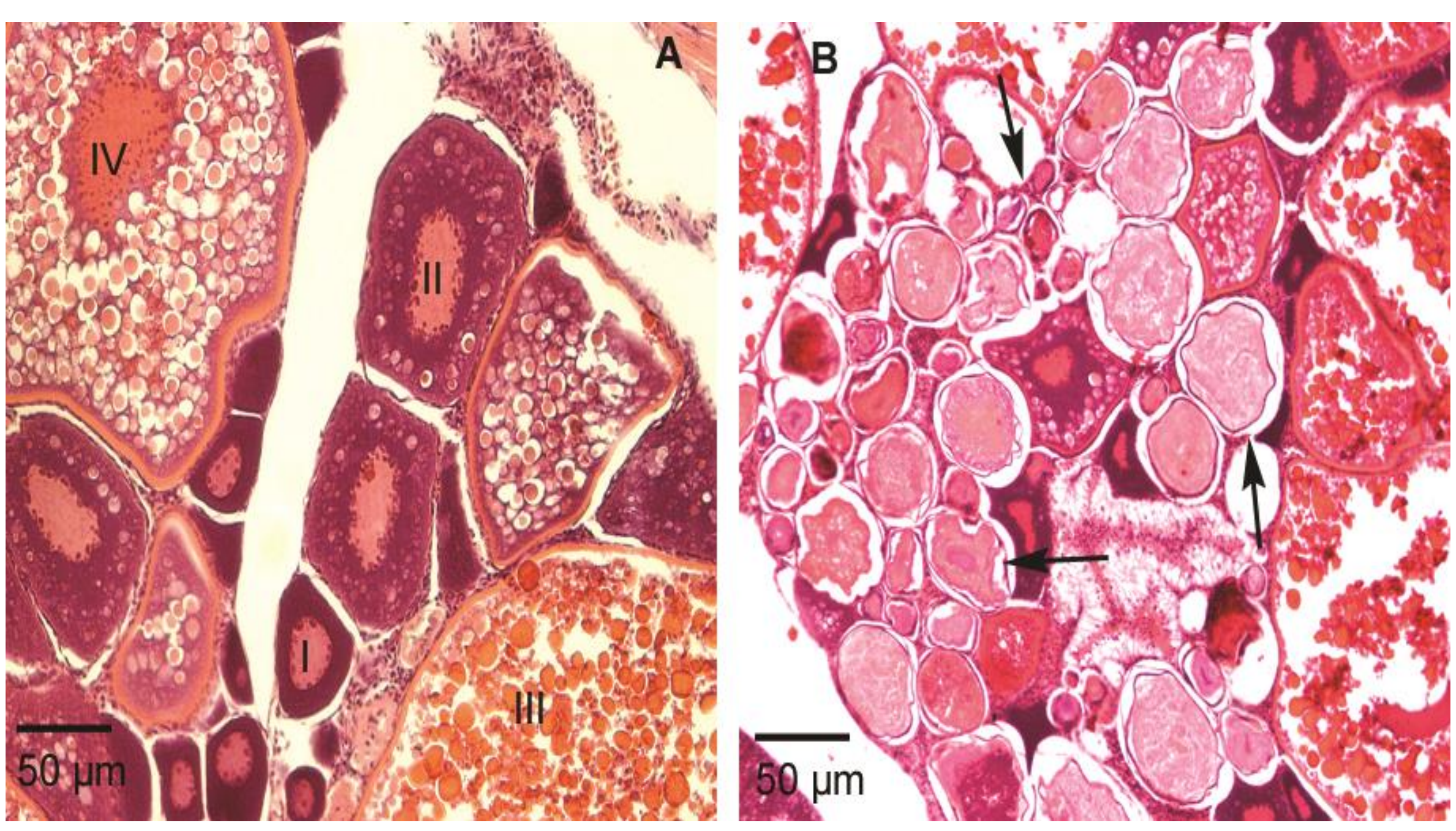
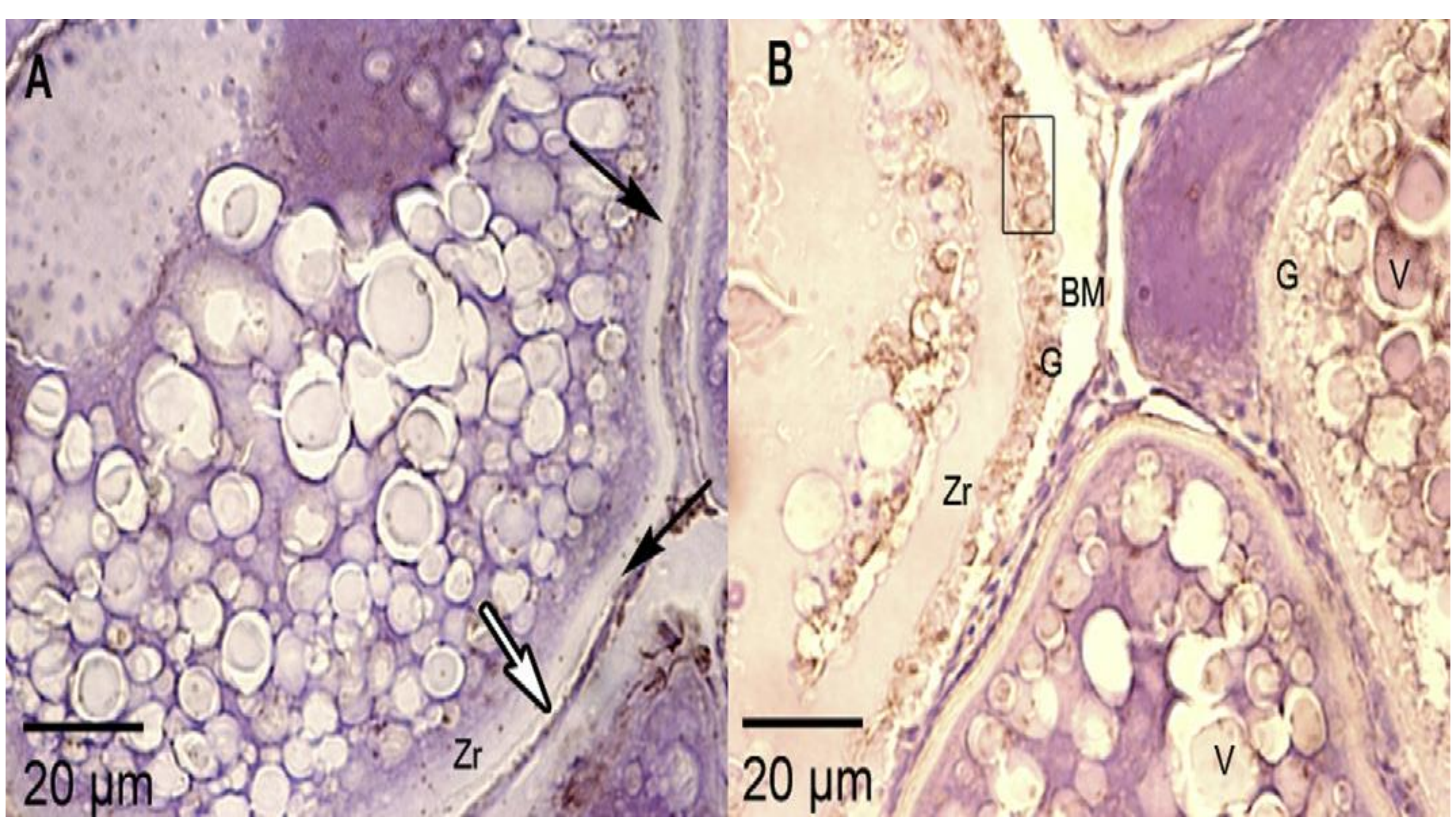

A
IV
II
I
III
50 µm
B
50 µm

**Figure 3.5: Apoptosis within the oocyte after pharmaceutical exposure.** A) Normal follicles showing uniform cellular structures within the granulosa (open arrow) and zona radiata (Zr). The thecal cell layer (solid arrow) is visible and free of any structural irregularities or apoptosis. B) Follicles of a female zebrafish exposed to 10 µg $L^{-1}$ of a mixture of pharmaceuticals, showing separation of the granulosal cell layers (G) from the basement membrane (BM) of the follicle, cuboidal and hypertrophic cells (rectangulated) within the granulosa, and ooplasmic vesciles (V). Tissues were incubated with the apoptotic maker diaminobenzidine (DAB), which appears brown under light microscopy, and then counter-stained with hematoxylin and eosin (60x).


A
Zr
20 µm
B
BM
G
G
V
Zr
G
V
20 µm

## REFERENCES

Andreozzi, R., Raffaele, M., Nicklas, P., 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere 50, 1319-1330.

Bendz, D., Paxéus, N.A., Ginn, T.R., Loge, F.J., 2005. Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Hoje River in Sweden. Journal of hazardous materials 122, 195-204.

Benotti, M.J., Brownawell, B.J., 2007. Distributions of Pharmaceuticals in an Urban Estuary during both Dry- and Wet-Weather Conditions. Environmental Science & Technology 41, 5795-5802.

Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder, S.A., 2008. Pharmaceuticals and Endocrine Disrupting Compounds in U.S. Drinking Water. Environmental Science & Technology 43, 597-603.

Bernet, D., Schmidt, H., Meier, W., Burkhardt-Holm, P., Wahli, T., 1999. Histopathology in fish: proposal for a protocol to assess aquatic pollution. Journal of Fish Diseases 22, 25-34.

Bowley, L.A., Alam, F., Marentette, J.R., Balshine, S., Wilson, J.Y., 2010. Characterization of vitellogenin gene expression in round goby (Neogobius melanostomus) using a quantitative polymerase chain reaction assay. Environ. Toxicol. Chem. 29, 2751-2760.

Brian, J.V., Harris, C.A., Scholze, M., Kortenkamp, A., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Marcomini, A., Sumpter, J.P., 2006. Evidence of Estrogenic Mixture Effects on the Reproductive Performance of Fish. Environmental Science & Technology 41, 337-344.

Brooks, B.W., Riley, T.M., Taylor, R.D., 2006. Water quality of effluent-dominated ecosystems: ecotoxicological, hydrological, and management considerations. Hydrobiologia 556, 365-379.

Brooks, S., Tyler, C.R., Sumpter, J.P., 1997. Egg quality in fish: what makes a good egg? Reviews in Fish Biology and Fisheries 7, 387-416.

Brun, G.L., Bernier, M., Losier, R., Doe, K., Jackman, P., Lee, H.B., 2006. Pharmaceutically active compounds in atlantic canadian sewage treatment plant effluents and receiving waters, and potential for environmental effects as measured by acute and chronic aquatic toxicity. Environ Toxicol Chem 25, 2163-2176.

Clara, M., Strenn, B., Gans, O., Martinez, E., Kreuzinger, N., Kroiss, H., 2005. Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a

membrane bioreactor and conventional wastewater treatment plants. Water Research 39, 4797-4807.

Clara, M., Strenn, B., Kreuzinger, N., 2004. Carbamazepine as a possible anthropogenic marker in the aquatic environment: investigations on the behaviour of Carbamazepine in wastewater treatment and during groundwater infiltration. Water Research 38, 947-954.

Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol Lett 142, 185-194.

Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicol Environ Saf 59, 309-315.

David, A., Pancharatna, K., 2009. Effects of acetaminophen (paracetamol) in the embryonic development of zebrafish, Danio rerio. J Appl Toxicol 29, 597-602.

Domagalski, J., Belitz, K., Furlong, E.T., 2007. Occurrence of pharmaceutical and other organic compounds in groundwater in ten regions of California, USA, American Geophysical Union Spring Meeting, Acapulco, Mexico.

Galus, M., Kirischian, N., Higgins, S., Purdy, J., Chow, J., Rangaranjan, S., Li, H., Metcalfe, C., Wilson, J.Y., 2013. Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. Aquatic Toxicology 132, 200-211.

Glassmeyer, S.T., Hinchey, E.K., Boehme, S.E., Daughton, C.G., Ruhoy, I.S., Conerly, O., Daniels, R.L., Lauer, L., McCarthy, M., Nettesheim, T.G., Sykes, K., Thompson, V.G., 2009. Disposal practices for unwanted residential medications in the United States. Environ Int 35, 566-572.

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.G.J., 2008. Evolutionary Conservation of Human Drug Targets in Organisms used for Environmental Risk Assessments. Environmental Science & Technology 42, 5807-5813.

Halling-Sorensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lutzhoft, H.C., Jorgensen, S.E., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment--a review. Chemosphere 36, 357-393.

Hignite, C., Azarnoff, D.L., 1977. Drugs and drug metabolites as environmental contaminants: chlorophenoxyisobutyrate and salicyclic acid in sewage water effluent. Life Sci 20, 337-341.

Huggett, D., Benson, W., Chipman, K., Cook, J., Gray, L., Kinter, L., Meyerhoff, R., Trudeau, V., 2005. Role of mammalian data in determining pharmaceutical responses in

aquatic species. Human pharmaceuticals assessing the impacts on aquatic ecosystems, 149-181.

Ings, J.S., Servos, M.R., Vijayan, M.M., 2011. Exposure to municipal wastewater effluent impacts stress performance in rainbow trout. Aquatic Toxicology 103, 85-91.

Kavanagh, R.J., Balch, G.C., Kiparissis, Y., Niimi, A.J., Sherry, J., Tinson, C., Metcalfe, C.D., 2004. Endocrine disruption and altered gonadal development in white perch (Morone americana) from the lower Great Lakes region. Environ Health Perspect 112, 898-902.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999−2000: A National Reconnaissance. Environmental science & technology 36, 1202-1211.

Kolpin, D.W., Skopec, M., Meyer, M.T., Furlong, E.T., Zaugg, S.D., 2004. Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing flow conditions. Science of The Total Environment 328, 119-130.

Lau, J., Butler, D., Schütze, M., 2002. Is combined sewer overflow spill frequency/volume a good indicator of receiving water quality impact? Urban Water 4, 181-189.

Li, H., Helm, P.A., Metcalfe, C.D., 2010. Sampling in the Great Lakes for pharmaceuticals, personal care products, and endocrine-disrupting substances using the passive polar organic chemical integrative sampler. Environ. Toxicol. Chem. 29, 751-762.

Lishman, L., Smyth, S.A., Sarafin, K., Kleywegt, S., Toito, J., Peart, T., Lee, B., Servos, M., Beland, M., Seto, P., 2006. Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. Sci Total Environ 367, 544-558.

Lister, A., Regan, C., Van Zwol, J., Van Der Kraak, G., 2009. Inhibition of egg production in zebrafish by fluoxetine and municipal effluents: A mechanistic evaluation. Aquatic Toxicology 95, 320-329.

Mennigen, J.A., Martyniuk, C.J., Crump, K., Xiong, H., Zhao, E., Popesku, J., Anisman, H., Cossins, A.R., Xia, X., Trudeau, V.L., 2008. Effects of fluoxetine on the reproductive axis of female goldfish (Carassius auratus). Physiol Genomics 35, 273-282.

Metcalfe, C.D., Chu, S.G., Judt, C., Li, H.X., Oakes, K.D., Servos, M.R., Andrews, D.M., 2010. Antidepressants and Their Metabolites in Municipal Wastewater, and Downstream Exposure in an Urban Watershed. Environ. Toxicol. Chem. 29, 79-89.

Metcalfe, C.D., Miao, X.S., Koenig, B.G., Struger, J., 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ Toxicol Chem 22, 2881-2889.

Mimeault, C., Trudeau, V.L., Moon, T.W., 2006. Waterborne gemfibrozil challenges the hepatic antioxidant defense system and down-regulates peroxisome proliferator-activated receptor beta (PPARbeta) mRNA levels in male goldfish (Carassius auratus). Toxicology 228, 140-150.

Parrott, J.L., Blunt, B.R., 2005. Life-cycle exposure of fathead minnows (Pimephales promelas) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. Environmental Toxicology 20, 131-141.

Perry, S.F., Shahsavarani, A., Georgalis, T., Bayaa, M., Furimsky, M., Thomas, S.L.Y., 2003. Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: Their role in ionic and acid-base regulation. Journal of Experimental Zoology Part A: Comparative Experimental Biology 300A, 53-62.

Robert, S.P., 1999. The Merck Manual, 17 ed. Merck Research Labs, Whitehouse Station, New Jersey.

Sacher, F., Lochow, E., Bethmann, D., Brauch, H.J., 1998. Occurrence of pharmaceutical active compounds in surface waters. Fachgruppe Wasserchemie in der Gesellschaft Deutscher Chemiker 90, 233-243.

Scheurer, M., Ramil, M., Metcalfe, C.D., Groh, S., Ternes, T.A., 2010. The challenge of analyzing beta-blocker drugs in sludge and wastewater. Anal Bioanal Chem 396, 845-856.

Schreck, C.B., Contreras-Sanchez, W., Fitzpatrick, M.S., 2001. Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture 197, 3-24.

Sedlak, D.L., Gray, J.L., Pinkston, K.E., 2000. Understanding Micro Contaminants in Recycled Water Environmental science & technology 34, 509A-515A.

Sema, I.U., Özlem, C., 2009. Atresia and Apoptosis in Preovulatory Follicles in the Ovary of Danio rerio (Zebrafish). Turkish Journal of Fisheries and Aquatic Sciences 9, 215-221.

Stackelberg, P.E., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Henderson, A.K., Reissman, D.B., 2004. Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water-treatment plant. Sci Total Environ 329, 99-113.

Tarrant, H., Mousakitis, G., Wylde, S., Tattersall, N., Lyons, A., Maloney, M., Llewellyn, N., 2008. Raised plasma vitellogenin in male wild brown trout (Salmo trutta) near a wastewater treatment plant in Ireland. Environ. Toxicol. Chem. 27, 1773-1779.

Thorpe, K.L., Cummings, R.I., Hutchinson, T.H., Scholze, M., Brighty, G., Sumpter, J.P., Tyler, C.R., 2003. Relative Potencies and Combination Effects of Steroidal Estrogens in Fish. Environmental Science & Technology 37, 1142-1149.

Tixier, C., Singer, H.P., Oellers, S., Muller, S.R., 2003. Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters. Environmental science & technology 37, 1061-1068.

Topp, E., Monteiro, S.C., Beck, A., Coelho, B.B., Boxall, A.B., Duenk, P.W., Kleywegt, S., Lapen, D.R., Payne, M., Sabourin, L., Li, H., Metcalfe, C.D., 2008. Runoff of pharmaceuticals and personal care products following application of biosolids to an agricultural field. Sci Total Environ 396, 52-59.

Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Woodling, J.D., Norris, D.O., 2008. Reproductive disruption in fish downstream from an Estrogenic wastewater effluent. Environmental Science & Technology 42, 3407-3414.

Wallace, R.A., Selman, K., 1981. Cellular and Dynamic Aspects of Oocyte Growth in Teleosts. American Zoologist 21, 325-343.

Wood, A.W., Van Der Kraak, G.J., 2001. Apoptosis and ovarian function: novel perspectives from the teleosts. Biol Reprod 64, 264-271.

Woodling, J.D., Lopez, E.M., Maldonado, T.A., Norris, D.O., Vajda, A.M., 2006. Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 144, 10-15.

# CHAPTER 4

## EFFECTS OF CHRONIC, PARENTAL PHARMACEUTICAL EXPOSURE ON ZEBRAFISH (*Danio rerio*) OFFSPRING

Reproduced with kind permission from the Journal of Aquatic Toxicology.
[Galus, M., Rangaranhan, S, Lai, A., Shaya, L., Balshine, S., Wilson, J.Y., 2014.
Aquat Toxicol 151, 124-134] © 2014

## ABSTRACT

In this study we explored how parental exposure to pharmaceuticals influences reproduction in offspring. Adult zebrafish (*Danio rerio*) were exposed for 6 weeks to 10 $\mu g\ L^{-1}$ of carbamazepine (CBZ) and gemfibrozil (GEM), two commonly prescribed drugs. Embryos were collected, reared in clean water until sexual maturity and then assessed for reproductive output, courtship, sperm function and organ histology. While 34% of the control pairs produced clutches, only 11% of the fish with CBZ exposed parents or 17% of the fish with GEM exposed parents produced clutches. Reciprocal crosses indicated that exposure in males had more profound reproductive effects. When a control $F_1$ male was crossed with either a $F_1$ female whose parents were CBZ or GEM exposed; no differences were observed in embryo production compared to controls. However, when a control $F_1$ female was crossed with either a CBZ or GEM $F_1$ male, 50% fewer embryos were produced. Male courtship was reduced in both CBZ and GEM $F_1$ fish but the deficits in courtship displays were drug specific. Compared to control males, the sperm from GEM $F_1$ males had shorter head lengths and midpieces whereas sperm from CBZ $F_1$ males had longer midpieces. Although it remains unclear how specifically these morphological differences influenced sperm velocity, the sperm from GEM $F_1$ males and from CBZ $F_1$ males swam faster than the sperm of control $F_1$ at 20s post activation. No significant differences were observed in the histology of the liver, kidney and gonads across treatment groups. These data are important as they show that chronic, low dose pharmaceutical exposure of parental fish is sufficient to cause significant reproductive effects in offspring.

## INTRODUCTION

The effects of parental contaminant exposure on offspring are a sorely understudied area of toxicology. Multi- or trans- generational effects are typically easier to study in laboratory using species with short life spans (Dietrich et al., 2010; Lürling et al., 2006; Péry et al., 2008) but can also be investigated with organisms that have lived for generations in polluted environments (Hontela et al., 1992; Nacci et al., 2002; Nye et al., 2007). In both cases, exposures are typically for the whole life cycle. Traditionally, life cycle assessments (LCA) utilize either a cradle-to-grave or cradle-to-gate approach (EPA, 1993). In both schemes, exposure is typically from the onset of fertilization until terminal sampling (full life cycle) or until 24 hr past sexual differentiation (partial life cycle, EPA, 1993). Rarely do studies extend beyond one generation. Yet, the effects documented in LCA may be caused during gamete production, embryogenesis, maternal deposition or uptake of contaminants into the egg, secondary sexual maturation or some combination of these important biological processes. The major challenge is that life cycle or multi-generational studies are difficult and time consuming to conduct and thus the number of studies that use vertebrates are limited. In a fast reproducing species such as zebrafish, these types of experiments are clearly possible. Given that many fish have migratory behaviors or distinct breeding grounds, quantifying the risks of parental versus embryonic contaminant exposures may be quite important and provide distinct data from traditional LCA approaches.

Studies that have looked at trans-generational impacts in aquatic species have generally focused on effects caused by estrogens (Greytak et al., 2010), estrogen like

compounds (Brennan et al., 2006) or polychlorinated biphenyls (PCBs) (Nacci et al., 2002). The number of studies that have explored the trans-generational impacts with pharmaceuticals is extremely limited. To date, only one multi-generational assessment involving fish (Parrott and Bennie, 2009) has been published and the majority of studies that have been conducted use invertebrates (Dzialowski et al., 2006; Kim et al., 2012; Ortiz-Rodríguez et al., 2012). In fish, maternal deposition of contaminants is one primary route of exposure for the developing embryo (Abrams and Mullins, 2009), however there are other mechanisms by which the progeny may be impacted by parental exposure. For example, fluctuations in circulating estrogen concentrations within the mother, during gametogenesis, have been linked to impaired growth rate and survival amongst offspring (Migliaccio et al., 1996). Furthermore, elevated cortisol levels, induced from behavioral stress during courtship in females, impacts the morphology and yolk-sac size of $F_1$ offspring in coral fishes (McCormick, 1998) and cichlid fish (Mileva et al., 2011). Male mediated effects are often associated to impairments with spermatozoa and DNA damage which has been linked to the incidence of skeletal abnormalities amongst $F_1$ (Devaux et al., 2011). Such studies provide evidence suggesting that parental exposure to endocrine disrupting compounds may impair fish populations over several generations.

Gemfibrozil (GEM) is a fibric acid derivative which is clinically prescribed to lower plasma triglycerides and total cholesterol (Spencer and Barradell, 1996). Although its exact mechanism of action is unclear, GEM is a peroxisome proliferator believed to elicit its effect by binding to the nuclear transcription factor peroxisome proliferator-activated receptor-alpha (PPARα) and promoting hepatic uptake and metabolism of free fatty acids

(Martin et al., 1997). Peroxisome proliferators have been heavily studied in mammalian systems and chronic exposure to them has been linked to the onset of hepatic carcinoma (Gonzalez, 1997) and efficiency reduction in the anti-oxidant defense system in rodents (O'Brien et al., 2001). Little work has been done to characterize the impact of GEM on fish, however goldfish *(Carassius auratus)* exposed to 1.5 μg $L^{-1}$ of GEM had decreased plasma testosterone (Mimeault et al., 2005) and adult zebrafish (*Danio rerio)* chronically exposed to 0.5 and 10 μg $L^{-1}$ of GEM show decreased reproductive output, atretic oocytes, and altered kidney histology (Galus et al., 2013b).

Carbamazepine (CBZ) is primarily prescribed as an anticonvulsant and mood stabilizer and is frequently used in the treatment of epilepsy and bipolar disorder (Brodie et al., 1995). CBZ is believed to have two target sites within vertebrates, triggering unique cellular responses. Its anticonvulsive properties are believed to occur through modulation of voltage gated sodium channels, maintaining the channels in an inactive state and reducing neuronal excitability (Brodie et al., 1995). However, its anti-depressive properties are believed to occur by lowering the turnover rate of arachidonic acid while keeping docosahexaeonic acid unaltered in brain (Rao et al., 2008). Japanese medaka (*Oryzias latipes*) acutely exposed to 6150 μg $L^{-1}$ of CBZ showed reduced feeding behaviour and swimming speed (Nassef et al., 2010). Sperm of the common carp (*Cyprinus carpio L.*), incubated for two hours with 2000 μg $L^{-1}$ of CBZ had reduced velocity (Li et al., 2010). Lastly, adult zebrafish chronically exposed to 0.5 and 10 μg $L^{-1}$ of CBZ showed decreased reproductive output, decreased plasma ketotestosterone in males, and altered kidney histology (Galus et al., 2013b).

Of the small number of studies that examined the effects of GEM and CBZ in fishes, most involved concentrations well above what would be considered environmentally relevant and most exposures were acute. Both GEM and CBZ have been detected in waste water effluent and surface waters with average effluent concentrations ranging from 0.84 – 4.76 μg $L^{-1}$ and 0.87 – 1.2 μg $L^{-1}$, respectively (Andreozzi et al., 2003; Kolpin et al., 2002; Metcalfe et al., 2003; Paxéus, 2004; Petrović et al., 2003; Zhang et al., 2008). Like other pharmaceuticals in the aquatic environment, CBZ and GEM enter receiving waters via waste water effluent discharge. Their release into surface waters is due to the low removal efficiency of these compounds in waste water treatment, which is usually between 40 – 50% for GEM and <20% for CBZ (Galus et al., 2013a; Metcalfe et al., 2003). Both GEM and CBZ have been detected in fish tissues such as muscle (0.4, 0.52 bio-concentration units) and adipose tissue (20.8, 4.16 bio-concentration units) (Zhang et al., 2010).

Testing of pharmaceuticals, such as CBZ and GEM, for effects on different life stages and across generations is necessary to ascertain if impacts from these pseudo-persistent contaminants are likely to have population level impacts. This will require chronic, low level exposures of adults and embryos as well as multi- or trans- generational and/or life cycle assessments. To date, most of the studies of CBZ and GEM have been conducted on adult fish; embryonic exposures and multi-generational impacts are almost non-existent. Previously we showed that parental exposure did not increase embryo mortality, abnormalities or altered hatching rates in zebrafish (Galus et al., 2013b). Yet, the direct exposure of zebrafish embryos to 0.5 and 10 μg $L^{-1}$ GEM and 0.5 μg $L^{-1}$ CBZ

increased mortality (Galus et al., 2013b). To our knowledge, only one study has conducted a full life cycle exposure with GEM and no such work has been done with CBZ. Fathead minnow (*Pimephales promelas*) exposed to 0.01 to 1 μg $L^{-1}$ GEM had normal embryogenesis, growth, survival, development, and egg production (Parrott and Bennie, 2009). In exposed fish, the only endpoints observed were a greater number of deformities in clutches laid by the $F_1$ (Parrott and Bennie, 2009).

Given the environmental presence and reproductive effects of CBZ and GEM in zebrafish, the purpose of this study was to investigate whether parental exposure to CBZ and GEM was sufficient to cause reproductive consequences to the first filial generation ($F_1$). We chronically exposed adult zebrafish to aqueous CBZ and GEM, at concentrations known to cause decreased reproduction in the parental generation, and then reared the offspring to sexual maturity in clean water. Our experimental procedure differed from conventional life cycle assessment methods as our exposure was primarily limited to the parents and the resultant embryos were exposed to aqueous GEM or CBZ for <1.5hr. At sexual maturity, $F_1$ zebrafish were examined for effects on reproduction (breeding success, clutch size), sperm function (morphology and velocity), male courtship behaviours (frequency & duration), and organ histology. These data are important to help understand if chronic, low dose pharmaceutical exposure to parental fish is sufficient to cause significant effects in offspring that have not been directly exposed to these compounds.

## METHODS

*Compounds Tested*

All pharmaceuticals were purchased from Sigma Aldrich (Sigma Aldrich, Canada). Gemfibrozil (GEM) and carbamazepine (CBZ) were dissolved in dimethyl sulfoxide (DMSO). The final nominal concentration of each pharmaceutical in exposure tanks was 0 control (CTL) and 10 μg $L^{-1}$. Nominal concentrations of pharmaceuticals were not verified in this study as compound preparation was identical to a prior study and the concentrations and stability of the compounds with this experimental design has been published elsewhere (Galus et al., 2013b). This concentration represents the highest expected is most environments; prior work has shown similar reproductive and histological effects of these drugs at this and a lower, more typical environmental concentration (Galus et al., 2013b). All tanks had a final concentration of DMSO at 0.004%.

*Adult Exposures*

Fish care is described in detail elsewhere (Galus et al., 2013b) and was in accordance to McMaster University's animal care policies and under approved animal use protocols. Briefly, fish were kept in our facility for one month to acclimatize and monitor reproductive output. Fish were sorted into a 1:1 sex ratio, based on a visual examination, and reproductive output was assessed 3-5 times per week for a minimum of 2 weeks prior to the onset of the experiment. Only fish from tanks with similar reproductive output were included in the experiment. All fish were between 8 to 9 months of age. Pairs in tanks were randomly assigned to a treatment group and moved to exposure tanks 3 days prior to the start of exposure in order to acclimate.

Triplicate, 10 L exposure tanks housed 30 zebrafish at a density of 4 fish $L^{-1}$. Water chemistry in exposure tanks did not differ from that of the holding tanks and was comprised of distilled water with 12 mg $L^{-1}$ sodium bicarbonate and 60 mg $L^{-1}$ sea salts (Instant Ocean, Spectrum Brands, USA). Tanks were recirculating, maintained at 28 - 29°C and pH of 7.0 - 7.5 with a 90% renewal every 3 days, for six weeks. Weekly measurements of pH, dissolved oxygen, conductivity, nitrate, nitrite, ammonia, alkalinity, and general hardness were conducted. Zebrafish were kept on a white, fluorescent, artificial light cycle of 14:10 hours light:dark and fed twice a day with a commercial flake food (Tetramin Tropical Flakes, Tetra, USA) and once per day with live, adult *Artemia* (GSL Brine Shrimp, USA).

After 4 weeks of exposure an egg trap was placed in each tank before the onset of light, the mechanical filters shut off and embryos were collected for 1.5hr. Embryos were placed in petri dishes containing E3 embryo rearing media (NaCl 5 μg $L^{-1}$, KCl 0.17 μg $L^{-1}$, $CaCl_2$ 0.33 μg $L^{-1}$ and $MgSO_4$ 0.33 μg $L^{-1}$) at a density of 50 embryos per plate and incubated at 28.5°C. Embryos were examined under a microscope at 6, 24, 48, 72 and 96 hours post fertilization (hpf). Embryos that were dead or abnormal and any post-hatch chorions were removed. Media was replaced as needed to maintain water quality. Embryos were reared to sexual maturity using a modified version of the rearing protocol outlined by Lawrence (2007). Specifically, larvae were fed 4x per day (2hr between feedings), starting at 5 days post fertilization (dpf), using a 50:50 mixture of spirulina (Aquatic eco-systems, USA) and grade 0 hatchfry encapsulon (Argent labs, USA) dissolved in 50 mL of distilled water and vortexed thoroughly. A pasteur pipette was used to provide 1 drop of food per plate and the mixture was evenly distributed throughout the $E_3$ media. Starting at 8 dpf, fry

were fed a combination of the spirulina and encapsulon mixture as well as live 1st instar *Artemia* larvae (GSL Brine Shrimp, USA). At 8 dpf, fry were transferred to 500 mL tanks containing 200 mL of the $E_3$ media at a density of 50 fish tank $^{-1}$. Between 11-14 dpf, fry were slowly transitioned from $E_3$ media to tank water, in order to minimize osmotic shock, by incrementally adding system water to the existing 200 mL of $E_3$ media. System water was added such that the final composition of tank contained 10, 25, 50, 80 and 100% system water each subsequent day. Special attention was provided to the thoroughness of water change out in the dishes and tanks, especially during feedings with the spirulina mixture, to prevent the formation of an algal bloom. From 15 dpf onwards, fry were fed 3x per day, twice with a combination of grade 0 and grade 3 hatchfry encapsulon and once with live 1st instar and adult *Artemia*. Fry remained in 500 mL tanks until their head width was ≥500 µm (~ 4-5 weeks post hatch), they were then transferred to holding tanks in a semi–recirculating system (28°C, pH 7-8, dissolved oxygen ≥87% and conductivity 470-455 µS) with ≥10% daily renewal (Aquatic habitats, FL, USA). Once fry reached 8 weeks post hatch they were transitioned to the adult diet as described above and raised until they were 6-7 months post hatch.

*Pairwise and Reciprocal Breeding*

The reproductive capabilities of the first filial ($F_1$) generation were assessed via mating crosses between pairs from within treatment and between pairs from reciprocal crosses across treatment groups. Reproductively mature males and females, between 8-9 months of age, were separated into holding tanks, transferred into pairwise breeding tanks (500 ml) overnight to assess reproduction, and then returned to their respective separate-

sex holding tanks and allowed 3 days recovery. Each pairwise tank held 1 male and 1 female either from within a treatment group (exposed male and exposed female) or across treatment groups (reciprocal cross: an exposed male and a control female or a control male and an exposed female). Fish were placed in a tank with an insert and divider to separate the sexes and kept overnight (12 hours prior to breeding) at 28.5°C. Before first light, dividers were removed and the pair was allowed to interact and breed for 1.5 hrs after onset of light; additional inserts at the bottom of the tank acted as a barrier between the zebrafish and the fertilized eggs to prevent clutch cannibalism. After 1.5 hours of breeding, fish were returned to their holding tank and the clutch, if present, was collected and counted. Embryos were transferred into petri dishes containing $E_3$ media and assessed for viability at 6hpf. In total, we made 75 pairwise within treatment crosses (CTL, CBZ and GEM) and reciprocal crosses between a $F_1$ CTL male + $F_1$ CBZ female; and a $F_1$ CTL male + $F_1$ GEM female. There was elevated aggression among the $F_1$ CBZ and GEM males that induced $F_1$ CTL female mortality thus, only 55 reciprocal crosses were completed for $F_1$ CBZ males with $F_1$ CTL females and 28 reciprocal crosses for $F_1$ GEM males with $F_1$ CTL females.

*Courtship Behaviour*

To accurately assess courtship behaviours, pairwise breeding was set up as described above. Before the onset of light all pairwise tanks were covered to maintain darkness until they could be filmed. The covers and dividers separating the pair were removed and courtship behaviours recorded for 10 minutes on a Panasonic Lumix digital camera, mounted on a tripod. Once 10 min elapsed, the assessed animals were set aside until all pairs were observed.

The videos were analyzed using VLC media player (v. 2.0.6, available at http://www.videolan.org/vlc/index.html) at 40% the original speed for total time (duration) and the number of occurrences (frequency) of characteristic male behaviours displays (Table 4.1). We focused on male behaviours because the male is more active during courtship and has a distinct set of behavioural displays that are used to entice the female to copulate. In contrast, the female does not display but simply accepts (follows) or declines (avoids) the male's displays (Cole and Smith, 1987; Darrow and Harris, 2004; Spence et al., 2008). The courtship behaviours were scored blind to the treatment group.

*Sperm Collection, Velocity and Morphology*

Sperm samples were collected from CTL, CBZ and GEM $F_1$ males to assess sperm morphology and swimming velocity. To maximize the volume of sperm collected, all sperm extractions were carried out between 8:30-10:30 am, the peak zebrafish breeding times. To stimulate sperm production, males were held separately from females and then transferred into a tank with reproductively mature, ripe females for one day. The next morning the males were transferred individually into 0.6 mM tricaine solution buffered to pH 7.0 with 1M Tris in clean fish water. Fish were anesthetized, removed and blotted with a kimwipe. The urogenital opening was thoroughly dried to prevent premature activation of sperm. Males were placed belly up in a sponge holder and a 10µl microcapillary tube was placed on the urogenital opening. Using a pair of fine forceps, the abdomen was gently massaged, in the direction of the cloacae, while suction was applied to the microcapillary tube. On average, males yielded 1-3µl of good quality sperm, identified through its milky white appearance, where as poor quality sperm was often translucent or transparent.

Animals recovered from anesthesia in clean fish water, were returned to the holding tanks and given 10 days rest to recover before the second collection. Sperm was collected a second time so that both sperm velocity and morphology could be measured in each animal. Sperm collections were completed with 91% survival.

We measured the height of sperm collected within the capillary tube as an estimate of sperm volume and qualitatively assessed the sperm quality by the white colour and opaqueness of the sample; the whiter and more opaque a sample was, the better the yield. Following collection, sperm was diluted in 100 – 400 µl of fish water. Dilution volume was determined based on both quality and volume of sperm collected. On average, a height up to 2mm was diluted between 100 – 200 ul and 2-5mm was diluted in 200 – 400 µl. The entire sample was then expelled into a welled slide (Fisher Scientific, Ottawa, Ontario), preplaced on a pre-focused Olympus CX41 light microscope (Olympus, America Inc, USA) and then covered with a coverslip. Video recording began a few seconds before the sperm was added to the welled slide to ensure that the sperm was recorded at the earliest time possible. Video capture was done at 200x magnification, under phase contrast, with a Prosilica EC-650 digital camera (Prosilica, Burnaby, British Columbia, Canada) mounted on the Olympus CX41 microscope (Olympus America Inc.). Videos were recorded using Astro IIDC (v. 4.04.00) software at 60 frames $s^{-1}$.

Sperm velocity was measured for 1 sec at 20, 30, 40 and 50 s post activation ( using a CASA (v.12) computer-assisted sperm analysis system (Hamilton-Thorne Research, Beverly, Maine, USA) with parameters optimized for zebrafish sperm (*Frames: 60 Hz, Contrast: 55, Cell size: 10, Optics magnification: 4.66*). The researcher was blind to the

treatment group when measuring sperm velocity. The median sperm velocity (VAP; median smooth path velocity) was calculated for all spermatozoa whose forward movement was traced for at least 0.33 s (≥20 frames) at each time period after activation.

The second sperm sample collected was expelled into 1ml of 10% neutral buffered formalin and thoroughly mixed to prevent clumping of spermatozoa. The samples were formalin fixed for 48h. Ten microliters of fixed sperm was diluted 1:1 with nanopure water on a glass microscope slide, covered with a coverslip and examined under phase contrast at 400x magnification using a Leica DMBL microscope (Leica Microsystems Inc., Buffalo, NY, USA). Using a mounted Prosilica EC-650 digital camera, pictures of 25 spermatozoa male$^{-1}$ were taken and analysed to the nearest 0.1 µm using National Institutes of Health's ImageJ software (v. 1.47j, available at http://rsb.info.nih.gov/ij/). Measurements of the sperm head length and width, mid-piece, mid-piece + flagellum were calculated by drawing a freehand line over each sperm section. Flagellum length was obtained by subtracting the length of midpiece form the combined mid-piece + flagellum measurement.

*Histology*

Methods for histological procedures were followed in accordance to (Galus et al., 2013b). Briefly, whole body (open body cavity) zebrafish were fixed in formalin for a minimum of 48 hr and then rinsed and stored in ethanol until decalcified with Richard Allen De-cal (Fisher Scientific, Ottawa, ON) following the manufacturer's protocol. Decalcified samples were then embedded in paraffin, serially sectioned in the parasagittal plane at 5µm and stained with hematoxylin and eosin Y (Richard Allen). Histopathological scores for each organ were assigned based on screening of at least 2 sections, in order to rule out

staining artifacts and staining differences. The slides were read blind and the scores for histopathology in the organs were adapted from (Bernet et al., 1999), as outlined in Galus (2013b).

*Statistics*

Normality of the data was determined using the Shapiro-Wilk's test. Differences in breeding success were analyzed using a series of Mann-Whitney rank sums tests, comparing each cross with the success of CTL pairs. Power analysis for clutch size was conducted using G*Power 3 (Dusseldorf University). Data for the frequency and duration of courtship displays was not normal, thus data was log transformed and significant differences determined using one-way ANOVA. We compared the courtship frequency and duration in control pairs to the rates observed in the reciprocal and other within treatment pairs. Due to variations in samples size; embryo production, sperm morphology and velocity were analyzed using a Mann-Whitney rank sums test. Differences in embryo production were compared to the CTL pair and differences in sperm measurements were compared to CTLs at each time point; not across time points. Histological data was analyzed based on the percent incidence of histopathology and mean pathological score, within each exposure and analyzed using a student's *t* test. All statistical tests, except for the power analysis, were conducted using SigmaPlot 11.0 (Systat Software). Following statistical analysis, pairwise differences were determined using a Holm-Sidak post-hoc test. Where applicable, all data was expressed as mean±SEM. The significance level was set at $p \leq 0.05$.

## RESULTS

*Embryo Production during Pairwise & Reciprocal Crosses*

Embryo production (i.e. viable embryos) was measured during 75 randomly paired breeding events for each within treatment group (i.e. both M and F from CTL, CBZ or GEM parents) and reciprocal crosses (i.e. only one gender from exposed parents and the other from control parents); except those of $F_1$ CTL F + CBZ M and $F_1$ CTL F + GEM M. CBZ and GEM $F_1$ males were overly aggressive towards $F_1$ CTL females, repeatedly impacted the abdomen through snout or tail fin contact. After breeding, females were left with swollen and bruised bellies and often did not recover from these injuries. There was 38% mortality during these reciprocal crosses but mortality was 0% in the CTL pairwise crosses. Thus, we stopped the reciprocal cross matings of $F_1$ CTL F + CBZ M and $F_1$ CTL F + GEM M after 56 and 28 breeding events, respectively. We did not observe elevated aggression leading to an increased female mortality in the within treatment crosses.

There were significant differences in reproductive success in the within treatment crosses between control and both CBZ and GEM groups (Figure 4.1). During matings involving $F_1$ CTL M + $F_1$ CTL F, a clutch was produced in 34% of pairs. Matings between $F_1$ CBZ M + $F_1$ CBZ F or $F_1$ GEM M + $F_1$ GEM F produced clutches in 11% and 17 % of matings, respectively. Taking into account both the number and size of the clutches, the mean embryo production for each pairwise cross was determined and CBZ and GEM groups had lower fecundity than CTL animals (Figure 4.2).

Reciprocal crosses were performed to isolate whether the decreased breeding with parental drug treatment was due to an effect on one or both genders. In these crosses, a $F_1$

CTL male was mated with a treated $F_1$ (CBZ or GEM) female or a treated $F_1$ (CBZ or GEM) male was mated with a $F_1$ CTL female. Breeding pairs involving CTL $F_1$ males had a similar fecundity to within CTL pairs (Figure 4.2) and there was no statistical difference between breeding success for $F_1$ CTL male + $F_1$ CTL female, $F_1$ CTL male + $F_1$ GEM female or $F_1$ CBZ female (Figure 4.1). Pairs involving a $F_1$ CBZ or $F_1$ GEM males had significantly lower fecundity than those involving a $F_1$ CTL male (Figure 4.2). Reciprocal crosses with either $F_1$ CBZ or $F_1$ GEM males mated with $F_1$ CTL females produced significantly fewer clutches (Figure 4.1).

For each pair, the clutch size (i.e. number of embryos produced in a single breeding event) was determined. Clutch sizes (Table 4.2) were highly variable and mean clutch sizes for matings with CTL $F_1$ males and females was 102±72 (mean±SD). There were no statistical differences in clutch size across the within treatment or reciprocal crosses. We conducted a power analysis using the variation in clutch sizes in our experiments and determined that 154 clutches would be needed in order to identify if there were differences in clutch size amongst treatment groups. Taking into consideration the breeding success in our crosses, 452 crosses would be required to produce this number of clutches in the crosses with CTL males and females.

*Courtship Behaviours*

As described above, zebrafish courtship is almost exclusively a male activity (Darrow and Harris, 2004). The male uses a series of distinct identifiable displays towards gravid females to lead them to a spawning site. All our control $F_1$ males courted in a typical zebrafish fashion beginning with an approach followed by leading and nudging the female

and then performed several figure eight or circle displays with fins raised and then this series would culminate with a quiver, a behaviour linked to sperm release before the sequence would begin again (Spence 2007, Stoltz & Neff 2006). The most common display for all males was approach, followed by lateral displays, then leading behaviour, quivers were performed most infrequently (Figure 4.4, Table 4.3). Control $F_1$ males paired with exposed females had completely typical behaviour in terms of both duration and frequency of courtship behaviour. Interestingly, differences were observed when we considered the treated male crosses (Figure 4.3, 4.4). Gemfibrozil (GEM) exposed males paired with control females spent less time leading females but approached and quivered with them more frequently than control $F_1$ males (Figure 4.4). Males in carbamazepine (CBZ) exposed pairs had more lateral displays but lower nudge rates compared to males in control pairings. No other behavioural differences were detected.

*Sperm Velocity & Morphology*

Sperm velocity was measured over a 1 s duration at 20, 30, 40 and 50 s post activation (time after activating spermatozoa with tank water). In all treatment groups, mean sperm velocity decreased by approximately 40-50% over the 50 seconds (Figure 4.5). At 20 and 30 s post activation, the sperm of CBZ $F_1$ males was significantly faster than sperm of control $F_1$ males (Figure 4.5). GEM $F_1$ males had sperm that swam significantly faster than sperm from $F_1$ CTL males at 20 s post-activation (Figure 4.5). Interestingly, the faster sperm from $F_1$ CBZ males possessed significantly longer mid-pieces than those measured from $F_1$ CTL male sperm. However, the faster swimming sperm from $F_1$ GEM swam faster despite having significantly shorter mid-piece than $F_1$ CTL sperm (Figure 4.6).

Other morphological differences were detected in the sperm of $F_1$ GEM and CBZ males. The sperm of $F_1$ GEM zebrafish had a significantly smaller head than sperm from control males, whereas CBZ $F_1$ zebrafish sperm had longer midpieces than $F_1$ CTL males (Figure 4.6).

*Histology*

Male and female $F_1$ zebrafish, raised from parents chronically exposed to 10 μg $L^{-1}$ of CBZ and GEM were screened for histopathologial damage in the kidneys, liver and reproductive organs. Animals from all treatments and sexes had no histological alterations in any organ examined (data not shown).

**DISCUSSION**

Exposure of adult zebrafish to 10 μg $L^{-1}$ CBZ and GEM caused a significant decline in fecundity (i.e. total embryos produced) with 6 weeks exposure (data not shown). This agrees with our previous research, where 6 weeks exposure to GEM and CBZ at 10 and 0.5 μg $L^{-1}$ reduced fecundity compared to unexposed fish (Galus et al., 2013b). In that study, CBZ and GEM not only reduced reproduction but exposed female zebrafish had elevated numbers of atretic oocytes and increased TUNEL positive staining, which indicated elevated apoptosis, in the ovaries (Galus et al., 2013b). This research suggested that CBZ and GEM had a direct effect on oocyte production (Galus et al., 2013b). Zebrafish exposed to 0.5 and 10 μg $L^{-1}$ CBZ had decreased 11-ketotestosterone (11-KT) levels suggesting altered sex steroid hormones levels (Galus et al., 2013b). Thus, exposure of adult fish appeared to impact zebrafish reproduction with direct effects on gonadal function and steroid levels (Galus et al., 2013b).

In our previous studies, parental exposure of zebrafish to either 0.5 and 10 μg $L^{-1}$ CBZ and GEM did not impact abnormality, mortality or hatching rate of the offspring during embryogenesis (up to 96 hpf), when reared in clean water after parental exposure (Galus et al., 2013b). In agreement with that data, we did not observe any alterations in abnormality, mortality or hatching rate of the offspring from parents exposed to 10 μg $L^{-1}$ CBZ or GEM, when reared in clean water (data not shown). In our current study, we continued to rear the $F_1$ offspring in clean water to sexual maturity to assess whether parental exposure impacted reproduction in $F_1$ offspring at sexual maturity.

To our knowledge, this is the first study to assess whether parental exposure to pharmaceuticals was sufficient to cause reproductive impacts in $F_1$ offspring that were not directly exposed to the compounds in any fish species. Traditional life cycle assessments either evoke a cradle-to-grave or cradle-to-gate approach (EPA, 1993). In both schemes, exposure is often from the onset of fertilization until terminal sampling (full life cycle) or until 24 hr past sexual differentiation (partial life cycle) (EPA, 1993). Generally, life cycle tests are conducted to evaluate potential chronic effects of chemicals on fish populations (Benoit, 1982). This study (Benoit, 1982), and other life cycle assessements, gives emphasis to potential population effects, mainly focusing on impacts relating to survival, developmental and reproduction, with the end goal often to determine a margin of safety or the No Observed Effect Concentration (NOEC). These kinds of studies often start exposure with $F_0$ (fertilized embryos) and end with $F_1$ fry or juveniles. Full life cycle assessments that aim to explain multi- or trans- generational effects typically expose and assess offspring for several generations (>$F_2$) (EPA, 1993; OECD, 2002). In these kinds of

tests, exposure starts with reproducing $F_0$ and continues until the $F_2$ are past sexual maturity (OECD, 2002). With multi-generational assessments, exposure impacts to the parents are recorded, however the focus is on effects with developmental and sexual differentiation to help understand how exposure can lead to multi-generational impacts (EPA, 1993). We have tested direct exposure of both CBZ and GEM on zebrafish embryogenesis and found that exposure to 0.5 and 10 μg $L^{-1}$ GEM and 0.5 μg $L^{-1}$ CBZ caused increased mortality, but did not affect abnormality rates (Galus et al., 2013b). We have not reared these animals past 96 hours post fertilization and cannot comment on any persistent impacts from direct embryonic exposure. Parrott and Bennie (2009) however did conduct a full life cycle assessment of 0.01 to 1 μg $L^{-1}$ of naproxen, gemfibrozil, diclofenac, ibuprophen, triclosan, salicylic acid, and acetaminophen in fathead minnow (*Pimephales promelas*) and observed no impacts in $F_1$ or $F_2$ offspring in that species.

Most traditional life cycle assessments provide continuous exposure of organisms throughout the experiment, typically from the onset of fertilization. Our methods differed from conventional life cycle assessment as the $F_0$ fish were adults and were exposed for only 4-6 weeks, $F_1$ offspring were immediately collected, and once the embryos were removed from the parental tank, the offspring were only held in clean water. Therefore the $F_1$ offspring in our study would have been exposed to aqueous GEM or CBZ for a maximum of 1.5 hr; enough time for the embryo to progress to the 16-cell stage maximum and on average embryonic exposure was less than 1.5 hr. There are three ways by which drug exposure may have impacted the $F_1$ offspring. First, the parental exposure may have altered the quality of egg and/or sperm. Second, maternal deposition of drug may have resulted in

exposure during embryogenesis. Third, embryos may have taken up aqueous drugs (while in the egg trap) immediately after being laid (but prior to the trap being removed from the parental tank). Although no uptake data is available for embryonic fish pertaining to CBZ and GEM, given the aqueous drug concentration, their bioconcentration factors and the small exposure window, we estimate that only a fraction of the compound would have been able to enter the embryo, and any effects are most likely attributable to parental exposures alone or due to maternal deposition in the egg. Maternal deposition of contaminants into eggs is known for lipophilic contaminants such as polychlorinated biphenyls (Westerlund et al., 2000) and organochlorines (Fisk and Johnston, 1998; Russell et al., 1998) however there are no studies that examine the deposition of pharmaceuticals into fish eggs. Both GEM and CBZ are lipophilic compounds, therefore it is possible that they could be deposited into the oocyte during vitellogenesis and production of yolk. However, as we did not measure GEM or CBZ concentration in the embryos, this route of embryonic exposure cannot be ruled out.

*Breeding Success & Embryo Production*

Our strategy for assessing reproductive effects in $F_1$ offspring was to first cross animals within treatment groups, i.e. male and female from control treatment or male and female from a single parental drug (CBZ or GEM) treatment. Out of 75 pairwise mating events, the reproductive success of the CTL pair was 34% (Figure 4.1). Zebrafish are asynchronous batch spawners, where an individual female does not spawn on daily basis, even if conditions are favorable (Breder and Rosen, 1966). On average, a female will spawn once every 1.9 days and even though the spawning cycle may be routine, the clutch size

can be highly variable ranging from 1 – 200 embryos (Eaton and Farley, 1974). In order to ensure our fish were in optimal breeding conditions, both males and females were fed to satiation three times a day with both commercial flake and live (artemia) foods, as well as separated from the opposite sex before the breeding trials to allow investment of nutrients to sperm and ovarian growth. Food quantity and quality is highly important in the reproductive output of fish (Bagenal, 1969). We typically fed 3 times a day but have a reduced feeding schedule (twice a day) on weekends. Fish in our facility breed less frequently at the beginning of the week (unpublished data), and we assume this is due to lowered weekend feedings. In this study, we completed pairwise breedings every day of the week and included days that were likely impacted by our feeding schedule to ensure we could complete large numbers of pairs within a reasonable time frame. Pairwise breeding success in our facility in unexposed, adult animals is typically 35-48%, and seems to depend on the batch and age of fish, days of the week we breed, and feeding schedule (unpublished data, J.Y. Wilson). Fish whose parents were exposed to CBZ and GEM had significantly lower breeding success (Figure 4.1) and fecundity (Figure 4.2), compared to controls. In all pairwise and reciprocal matings involving $F_1$ males from exposed parents, reproductive success was below 15% (Figure 4.1) and mean fecundity was reduced by at least 50% (Figure 4.2) when compared to the control pairs. Yet, the reciprocal crosses involving control males with females whose parents had drug exposure were not different from unexposed parents control crosses, in terms of either breeding success (Figure 4.1) or fecundity (Figure 4.2). The consistent low level of reproductive success and mean embryo

production, in matings involving males from exposed parents but not females from exposed parents, suggest that parental exposure impacted males more than females.

The clutch of a female zebrafish can be highly variable ranging from 1-200 embryos (Eaton and Farley, 1974). Mean clutch sizes were not different between offspring from exposed parents and control offspring with unexposed parents (Table 4.2). However given the small number of clutches produced from the crosses involving males from treatment groups, there was too little statistical power needed to distinguish any potential effects. Based on the variance in clutch size observed in our experiment, a power analysis was conducted and revealed that 154 clutches would have been needed in order to uncover differences across treatment groups. Given rather low reproductive success observed in this study (a maximum around 30%), we would have needed to conduct at least 452 crosses to produce 154 clutches. It is therefore extraordinarily difficult to assess impacts on clutch size and clutch size is likely a difficult endpoint in zebrafish.

Interestingly, in the $F_1$ GEM M + CTL F reciprocal cross, the GEM male was only able to stimulate the female to successfully produce a clutch once out of 28 attempted mating events. In this treatment, along with CBZ male + CTL female, we had to terminate our pairwise matings prior to completing the 75 crosses achieved in other treatment groups due to an increase in male aggression that resulted in serious female injury and mortality. Mate choice amongst female zebrafish may be linked to olfactory cues as Gerlach (2006) has shown that females exposed to male pheromones produce 17% more viable embryos than unexposed females. Although the exact pheromones involved are unknown, the male derived F prostaglandin is known to stimulate follicular ripeness in goldfish (Sorensen et

al., 1995) and other teleosts (Goetz and Garczynski, 1997). In order for successful reproduction to occur, both males and females need to find a sexually mature individual of the opposite sex possibly through the use of pheromonal cues, and a sequence of courtship displays must be performed to ensure successful clutch production (Gerlach, 2006). Considering the increased aggression identified with the males from the treatment groups and the fact that courtship behaviour is primarily completed by male zebrafish, courtship or breeding behaviour was investigated.

*Courtship Behaviour*

Courtship behaviour amongst zebrafish is primarily conducted by males alone who display actively to stimulate the female to release a clutch (Darrow and Harris, 2004). Females will accept or decline the male courtship display but do not appear to alter their behaviour or produce any courtship behaviour of their own (Darrow and Harris, 2004). There are five characteristic behaviours previously identified in courtship and reproduction of zebrafish; approach, leading, lateral, nudge and quiver (Darrow and Harris, 2004). Male zebrafish perform this series of identifiable behaviours often starting with an interaction display (approach) and ending with quiver; as this behaviour is linked to release of sperm (Darrow and Harris, 2004). It is not well known if the behaviours must occur in a specific order (Darrow and Harris, 2004). We did notice that control $F_1$ males always initiate courtship by approaching and then leading the female, before engaging in physical contact behaviours such as nudges. During pairwise and reciprocal crosses, males were analyzed for both the duration (total time spent; Figure 4.3 & Table 4.3) and frequency (number of individual displays; Figure 4.4, Table 4.3) of the five characteristic male behaviours in the

initial 10 minutes after first light. Darrow and Harris (2004) have extensively examined zebrafish courtship displays and have shown that egg laying peaks within the first 10 min of courtship and occurs infrequently beyond 30 min. Although zebrafish can engage in courtship behaviours and clutch laying beyond the first 10 min, these activities sharply decline at 30 min post first light, and occur seldom beyond 70 min (Darrow and Harris, 2004). By recording the most robust time in which zebrafish are actively courting, we should have captured most of the breeding displays that would occur between pairs.

While courtship displays have been qualitatively described, to our knowledge, no study has documented the frequency or duration of specific behaviours during courtship. Yet, this is clearly needed to determine how exposure/treatment affects courtship behaviour. Males spent significantly more time approaching the female than any other behaviour (Table 4.3); control males had a lower frequency of quiver compared to any other behaviour (Table 4.3). While quantitative studies of courtship behaviour are not established for zebrafish, the characteristic courtship behaviours in other fish species have been well documented (Breder and Rosen, 1966; Cole and Smith, 1987; Darrow and Harris, 2004). Based on the qualitative research in zebrafish and quantitative research in other fish species, we can conclude that the control animals within our study were displaying typical courtship behaviours.

Successful reproduction requires a suite of behaviours and interactions ranging from spawning site selection, to courtship and post natal investment (Potts, 1984). Toxicant exposure could potentially interfere with the order or timing of appropriate reproductive behaviours and disrupt mating. In our study, GEM males were observed to spend less time

in leading, but invested a higher frequency in approached and quivered at higher frequencies compared to control pairings. CBZ males performed more lateral displays, but fewer nudges when compared to control males. Similar effects have been reported in Japanese medaka (*Oryzias latipes*) that were exposed to 25 μg $L^{-1}$ of 4-tert-octyphenol until 6 months post fertilization and showed a decreased frequency of approach and leading (Gray et al., 1999). Spawning goldfish (*Carassius auratus*) (Bjerselius et al., 2001) and adult Japanese medaka (Oshima et al., 2003) exposed to 1 μg $L^{-1}$ of 17β-estradiol for 28 days, both showed reduced frequency of leading and nudge. Long term exposure to estrogens has the ability to alter circulating levels of other hormones, including those which are key for normal reproduction (Nash et al., 2004). Bell (2001) examined the effects of estrogenic compounds on the courtship behaviour of stickleback (*Gasterosteus aculeatus*) and found a positive correlation between courtship behaviour and breeding success. Collectively, these studies suggest that estrogenic compounds can impact normal courtship behavior; and in our study the pharmaceuticals CBZ and GEM appeared to impact male courtship in $F_1$ offspring. Interestingly, CBZ has been shown to alter hormone levels in adult fish (Galus et al., 2013b; Li et al., 2010). Although the exact mechanism impacting the duration or frequency of spawning behaviours observed in our study is unknown, it is possible that altered steroidogenesis was the mechanism. We did not have sufficient numbers of animals to verify if parental exposure altered hormone levels in $F_1$ offspring.

*Sperm Morphology & Velocity*

The relationship between spermatozoa form and function has been heavily debated and studied in a broad range of species (Gage et al., 2002; Malo et al., 2006; Parker, 1993; Snook, 2005). Some of the above studies suggest that the morphology of the sperm is not always indicative of its velocity or swimming performance. However, sperm morphology and velocity have been shown to be good indicators of male fertilization rates (Casselman et al., 2006). The sperm of GEM $F_1$ males was found to have a shorter head and mid-piece length (Figure 4.5), whereas CBZ $F_1$ males had a larger mid-piece when compared to sperm from control males (Figure 4.5). We also analyzed sperm velocity and both GEM and CBZ sperm swam faster at 20 seconds post activation; CBZ was also faster 30 seconds post activation compared to CTLs (Figure 4.6). Despite there being conflicting points of view whether sperm form is linked to function, the changes observed in GEM and CBZ $F_1$ morphology could explain the alterations observed in velocity.

The spermatozoa of CBZ males possessed a larger midpiece (Figure 4.5) than that of CTL sperm. The midpiece is the region of sperm which is rich in mitochondria and produces all the ATP needed for spermatozoa propulsion (Ruiz-Pesini et al., 1998). It is plausible that with a larger midpiece, there was a higher abundance of mitochondria which in turn were able to produce a higher proportion of ATP resulting in the increased swimming speed observed over CTL sperm (Figure 4.6). In a comparative study between the sperm of many species of New World blackbirds, it was found that spermatozoa with a larger midpiece had a higher swimming velocity over those with a smaller midpiece (Lüpold et al., 2009). To our knowledge, the impacts of CBZ exposure on fish sperm have

not been studied before. Humans treated with therapeutic doses of CBZ show similar changes in sperm morphology (Isojärvi et al., 2004) and velocity (Pack, 2005) to those that were observed in our study.

Spermatozoa of GEM males had a shorter midpiece along with smaller head length than CTL animals (Figure 4.5). As discussed above, the midpiece is the portion of the sperm which generates the energy to propel the sperm forward. Although GEM sperm had a shorter midpiece than CTL sperm, they possessed a higher swimming velocity at 20s post activation (Figure 4.6). In theory, it may seem counterintuitive for a sperm with a reduced midpiece to outperform a sperm with a larger midpiece, however GEM sperm also possessed a smaller head length than that of CTL sperm (Figure 4.5). It is plausible that the morphological changes to midpiece, coupled with those of the head, could have reduced the surface area of the spermatozoa, in turn reducing the resistance associated with swimming and therefore contributed to faster swimming speed observed (Figure 4.5, 4.6). Although there are no studies which definitively correlate multiple changes in sperm morphology to swimming speed, it has been shown that a smaller head:flagellum ratio results in an overall increase in sperm velocity amongst passerine birds (Lüpold et al., 2009). Work with clofibric acid, a drug with a similar mode of action to GEM, has shown that exposure of fathead minnows to 1000 μg $L^{-1}$ reduces sperm count and velocity (Runnalls et al., 2007). Much like clofibric acid, GEM modulates androgens, and spermatogenesis is a heavily androgen dependant process (Schulz et al., 2001).

Alterations in sperm morphology and velocity may suggest impacts on embryo fertilization rate as well, yet our data does not necessarily suggest this. We did not see an

increase in unfertilized embryos in crosses with CBZ or GEM $F_1$ males. Indeed, most of the embryos were fertilized. Warner (1997) suggests that small fish species do not release excessive amounts of sperm during courtship, therefore any alterations in sperm quality or quantity could directly impact fertilization rates. In our study, breeding success was lowered in those crosses with CBZ and GEM $F_1$ males but fertilization rates were not altered. It may be that larger numbers of clutches are needed to document subtle differences in fertilization success.

Although the exact mechanism by which parental exposure can impact the physiology in offspring is unknown, it is possible that chronic pharmaceutical exposure may result in permanent changes to the target cells, allowing the effects of the drug to persist long after the exposure has stopped (Csoka and Szyf, 2009). Maternal deposition into the egg (Fisk and Johnston, 1998; Russell et al., 1998) is a possible mechanism for ensuring persistent exposure; even after the source contaminant has been removed. Transcriptional changes (Nakari and Erkomaa, 2003), or DNA methylation (Mhanni and McGowan, 2004), as a result of the embryonic exposure or during gamete production are possible mechanisms by which parental exposure could result in $F_1$ offspring effects. However, which process and which target cells are impacted are dependent on mechanism of action of the specific pharmaceutical. We have not yet assessed transcriptional changes in zebrafish embryos or parental gamete cells, after CBZ and GEM exposure. Transcriptomic studies will be necessary to understand if transcriptional changes are present and persistent to sexual maturity in $F_1$ offspring.

*Conclusion*

The results of this study demonstrate that chronic exposure of adult zebrafish to GEM and CBZ is sufficient to reduce breeding success and fecundity, alter courtship behaviours and sperm morphology and velocity in $F_1$ offspring reared in clean water. Thus, parental exposure of fish to pharmaceuticals may be sufficient to cause reproductive effects even if offspring are not environmentally exposed to the compound themselves. $F_1$ offspring from exposed parents showed reduced reproductive output but this effect appeared to be primarily related to males. Females from exposed parents were successful at breeding with control males. Male courtship displays were impacted by parental exposure to both CBZ and GEM, suggesting that males from exposed parents were not able to successfully court a female. Finally, sperm morphology and velocity was impacted by parental exposure to CBZ and GEM. Although we are unable to describe the mechanism of action behind these effects, we have shown that chronic parental exposure is sufficient enough to impact reproductive systems in offspring. Future research is now needed to explore hormone levels in $F_1$ offspring and to examine gene expression changes in brains and gonads of male $F_1$ offspring. Such results will help elucidate the mechanism by which parental GEM and CBZ exposures selectively impact male offspring.

**ACKNOWLEDGEMENTS**

The authors would like to thank Natalie Sopinka and Dr. Trevor Pitcher for their discussion regarding CASA operation. We would also like to thank Susan Marsh-Rollo for providing us access to the equipment and space to conduct the CASA measurements. Funding was provided by the Natural Sciences and Engineering Research Council of Canada in the form of a Discovery Project and Accelerator Grant to J.Y.W and the Ontario Ministry of Technology and Innovation in the form of an Early Researcher Award to J.Y.W. The Canadian Foundation for Innovation and Ontario Ministry of Research and Innovation provided infrastructure support to J.Y.W.

**Table 4.1: Pictogram describing adult courtship behaviours amongst zebrafish.** The primary male courtship displays in zebrafish are depicted. The letter 'M' next to the fish denotes the male and the arrows show a typical path the male would take during a particular display. The images used were adapted from Darrow and Harris (2004).

| Behavior | Description | Pictogram |
| --- | --- | --- |
| ***Chasing*** | Rapid swimming movement directly toward the female terminating 2-3 cm before contact | Chase M |
| ***Leading*** | Rapid straight line, zig-zag, or figure 8 pattern in front or around the female attempting to lead her to a spawning site | M Leading |
| ***Lateral display*** | Parallel positioning to the female and display of caudal fins | Lateral M |
| ***Nudging*** | Contact with the snout to position the female in direction of the spawning site | Nudge M |
| ***Quivering*** | Parallel positioning to the female delivering a series of small amplitude, but high frequency undulations against her body | M Quiver |

**Table 4.2: Mean clutch size in F1 zebrafish during pairwise and reciprocal matings.** Male and female $F_1$ zebrafish were placed in pairwise tanks, within treatment and in reciprocal crosses. Pairs were given until 1.5 hours after onset of light to breed; after which the presence or absence of a clutch was noted. There was no variation amongst clutch size between pairs. CBZ is carbamazepine, GEM is gemfibrozil, CTL is control, M is male and F is female. The pairing of GEM M + CTL F produced a single clutch in all 75 breeding events, therefore the standard deviation (SD) is not available (NA).

| | **CTL M + CTL F** | **CTL M + CBZ F** | **CBZ M + CTL F** | **CBZ M + CBZ F** | **CTL M + GEM F** | **GEM M + CTL F** | **GEM M + GEM F** |
|---|---|---|---|---|---|---|---|
| **Mean Clutch ± SD** | 102 ± 72 | 155 ± 113 | 73 ± 58 | 93 ± 82 | 122 ± 108 | 68 ± NA | 171 ± 144 |

**Table 4.3**. **Differences in duration and frequency of breeding behaviours within CTL pairs**. Male and female $F_1$ zebrafish were placed in pairwise tanks maintained in the dark with a tank divider. See Table 1 for a description and pictogram of the behaviours. A one-way ANOVA was conducted on log transformed data and significant differences between behaviours were analyzed within the CTL pair. Behaviours that share a common letter are not statistically different from one another. $p \leq 0.05$. CTL is control, M is male and F is female. Data is represented as mean ± standard deviation (SD).

| CTL M + CTL F | Approach | Leading | Lateral | Nudge | Quiver |
|---|---|---|---|---|---|
| **Time** | 468 ± 144$^{A}$ | 96 ± 169$^{BC}$ | 55 ± 62$^{CD}$ | 32 ± 31$^{CD}$ | 9 ± 26$^{D}$ |
| **Frequency** | 31 ± 14$^{AC}$ | 17 ± 13$^{AB}$ | 138 ± 141$^{C}$ | 51 ± 59$^{AC}$ | 1.7 ± 3$^{D}$ |

**Figure 4.1: Breeding success in $F_1$ zebrafish during pairwise and reciprocal matings**. Male and female $F_1$ zebrafish were placed in pairwise tanks, within treatment and in reciprocal crosses. Pairs were given until 1.5 hours after onset of light to breed; after which the presence or absence of a clutch was noted. Breeding success was based on 75 pairwise crosses for each within and across mating except for reciprocal crosses with CBZ and GEM males. CBZ and GEM males were aggressive and induced female mortality when paired with control females and only 55 and 28 reciprocal crosses were completed for CBZ male and control female and GEM male and control female groups, respectively. Significant differences were determined using a Mann-Whitney rank sums test. Letters denote significant differences from the within CTL pair. $p \leq 0.05$. CBZ is carbamazepine, GEM is gemfibrozil, CTL is control, M is male and F is female.

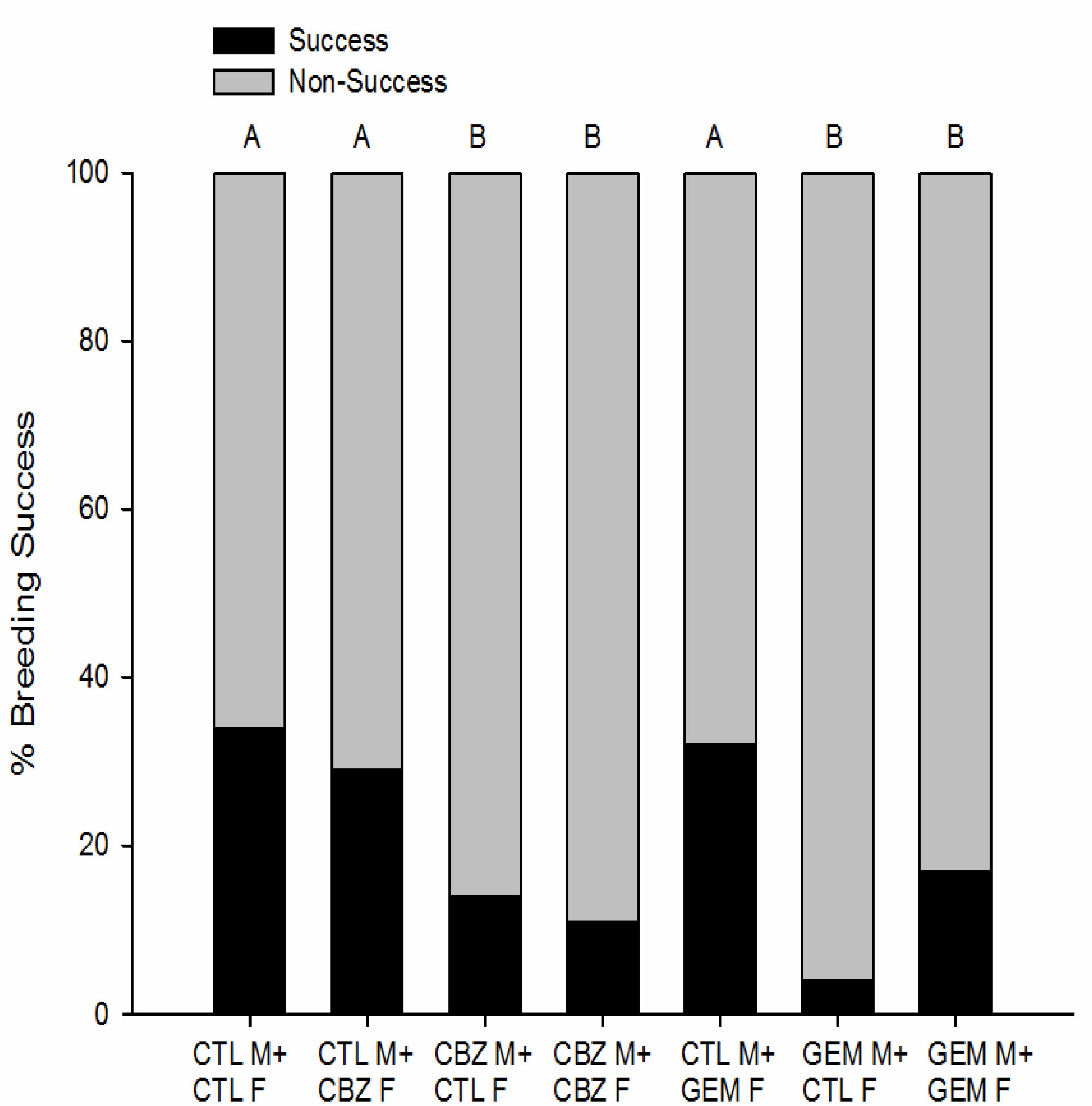

Success
Non-Success
A
A
B
B
A
B
B
100
80
60
40
20
0
% Breeding Success
CTL M+
CTL F
CTL M+
CBZ F
CBZ M+
CTL F
CBZ M+
CBZ F
CTL M+
GEM F
GEM M+
CTL F
GEM M+
GEM F

**Figure 4.2: Mean embryo production in $F_1$ zebrafish during within treatment and reciprocal matings**. Male and female $F_1$ zebrafish were placed in pairwise tanks, within treatment and in reciprocal crosses. Pairs were given until 1.5 hours after onset of light to breed; after which embryos were collected and counted. Mean embryo production was based on 75 pairwise crosses for each within and across mating except for reciprocal crosses with CBZ and GEM males. CBZ and GEM males were aggressive and induced female mortality when paired with control females and only 55 and 28 reciprocal crosses were completed for CBZ male and control female and GEM male and control female groups, respectively. Significant differences were determined using a Mann-Whitney rank sums test. Letters denote significant differences from the within CTL pair. $p \leq 0.05$. CBZ is carbamazepine, GEM is gemfibrozil, CTL is control, M is male and F is female.

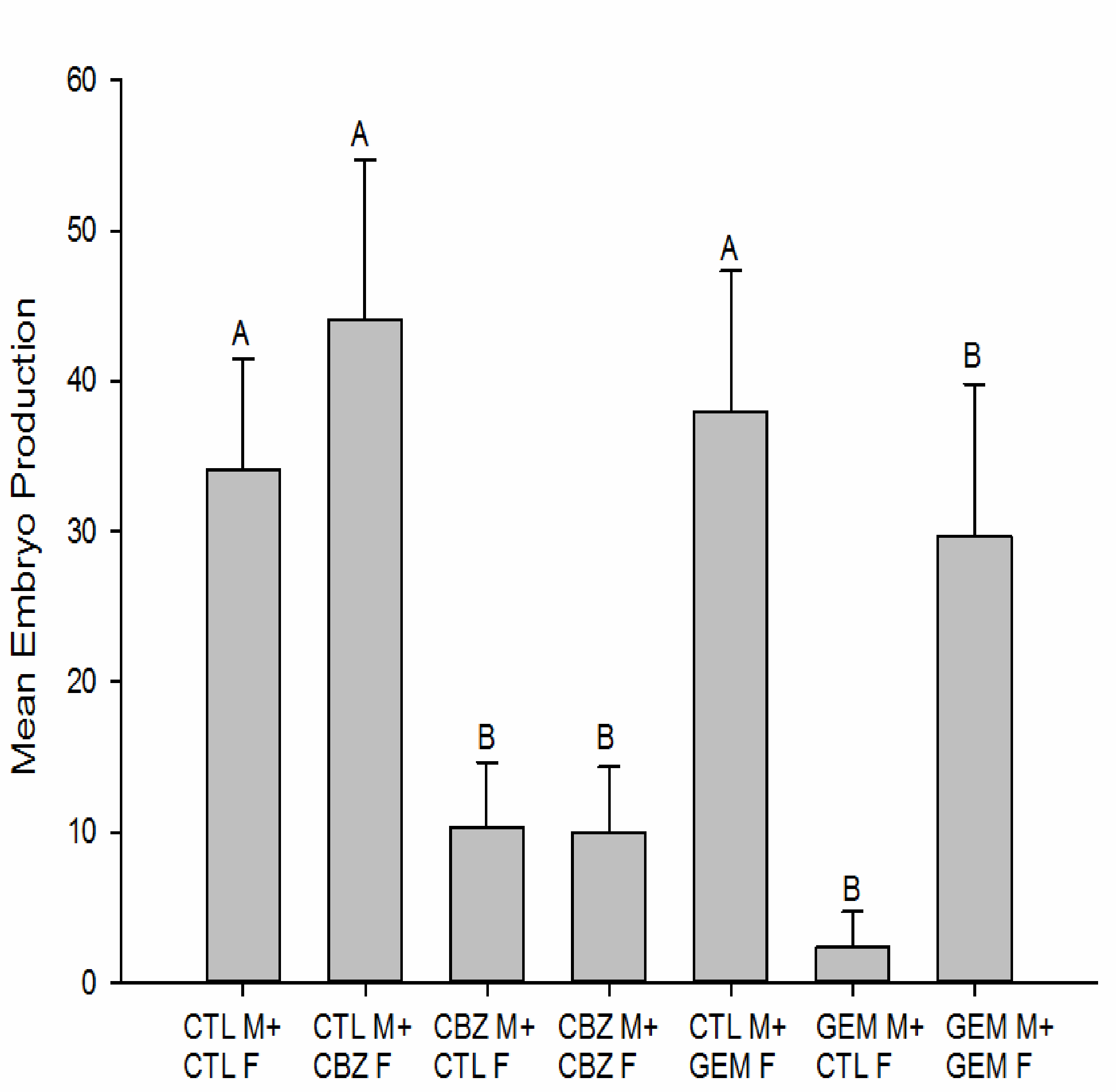

Mean Embryo Production
60
50
40
30
20
10
0
A
A
B
B
A
B
B
CTL M+
CTL F
CTL M+
CBZ F
CBZ M+
CTL F
CBZ M+
CBZ F
CTL M+
GEM F
GEM M+
CTL F
GEM M+
GEM F

**Figure 4.3: Duration of male courtship behaviours during $F_1$ mating**. Male and female $F_1$ zebrafish were placed in pairwise tanks, within treatment and in reciprocal crosses and maintained in the dark with a tank divider. Courtship behaviours were recorded immediately after removing divider at first light on a Panasonic Lumix digital camera for 10 min. The videos were then analyzed for the amount of time (in seconds) characteristic male behaviours were displayed. The behaviours examined included approach, leading, lateral, nudge and quiver (Darrow and Harris, 2004). See Table 1 for a description and pictogram of the behaviours. A one-way ANOVA was conducted on log transformed data and significant differences between behaviours were across all treatments for individual behaviours only. When comparing across treatments, the asterisk denotes difference in that behaviour compared to that seen in the CTL crosses. $p \leq 0.05$. CBZ is carbamazepine, GEM is gemfibrozil, CTL is control, M is male and F is female.

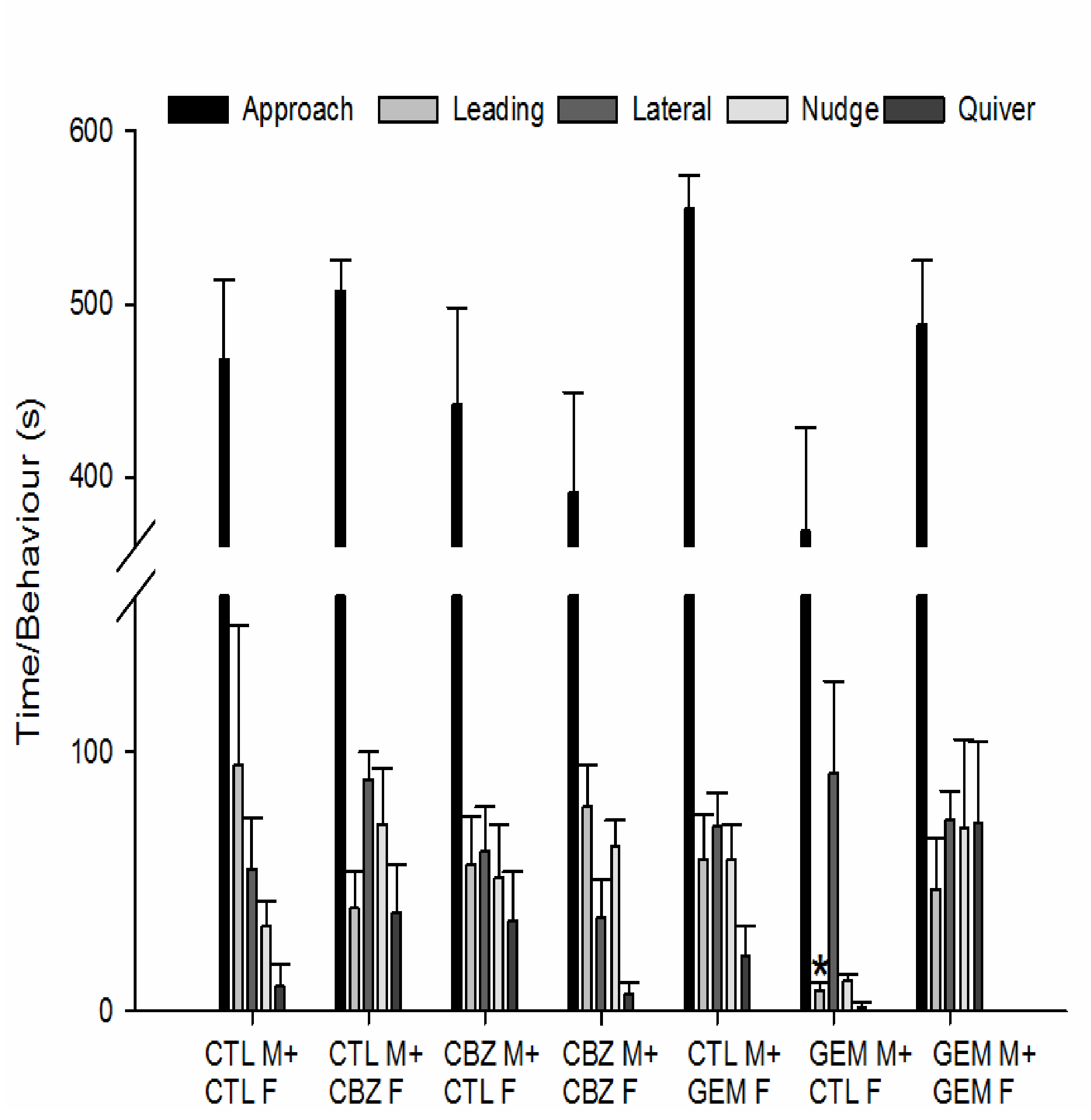

Approach
Leading
Lateral
Nudge
Quiver
Time/Behaviour (s)
600
500
400
100
0
*
CTL M+
CTL F
CTL M+
CBZ F
CBZ M+
CTL F
CBZ M+
CBZ F
CTL M+
GEM F
GEM M+
CTL F
GEM M+
GEM F

**Figure 4.4: Frequency of male Courtship behaviours during $F_1$ mating.** Male and female $F_1$ zebrafish were placed in pairwise tanks, within treatment and in reciprocal crosses and maintained in the dark with a tank divider. Courtship behaviours were recorded immediately after removing divider at first light on a Panasonic Lumix digital camera for 10 min. The videos were then analyzed for the number characteristic male behaviours displayed during the 10 minutes of breeding. The behaviours examined included approach, leading, lateral, nudge and quiver (Darrow and Harris, 2004). See Table 1 for a description and pictogram of the behaviours. A one-way ANOVA was conducted on log transformed data and significant differences between behaviours were across all treatments for individual behaviours only. When comparing across treatments, the asterisk denotes difference in that behaviour compared to that seen in the CTL crosses. $p \leq 0.05$. CBZ is carbamazepine, GEM is gemfibrozil, CTL is control, M is male and F is female.

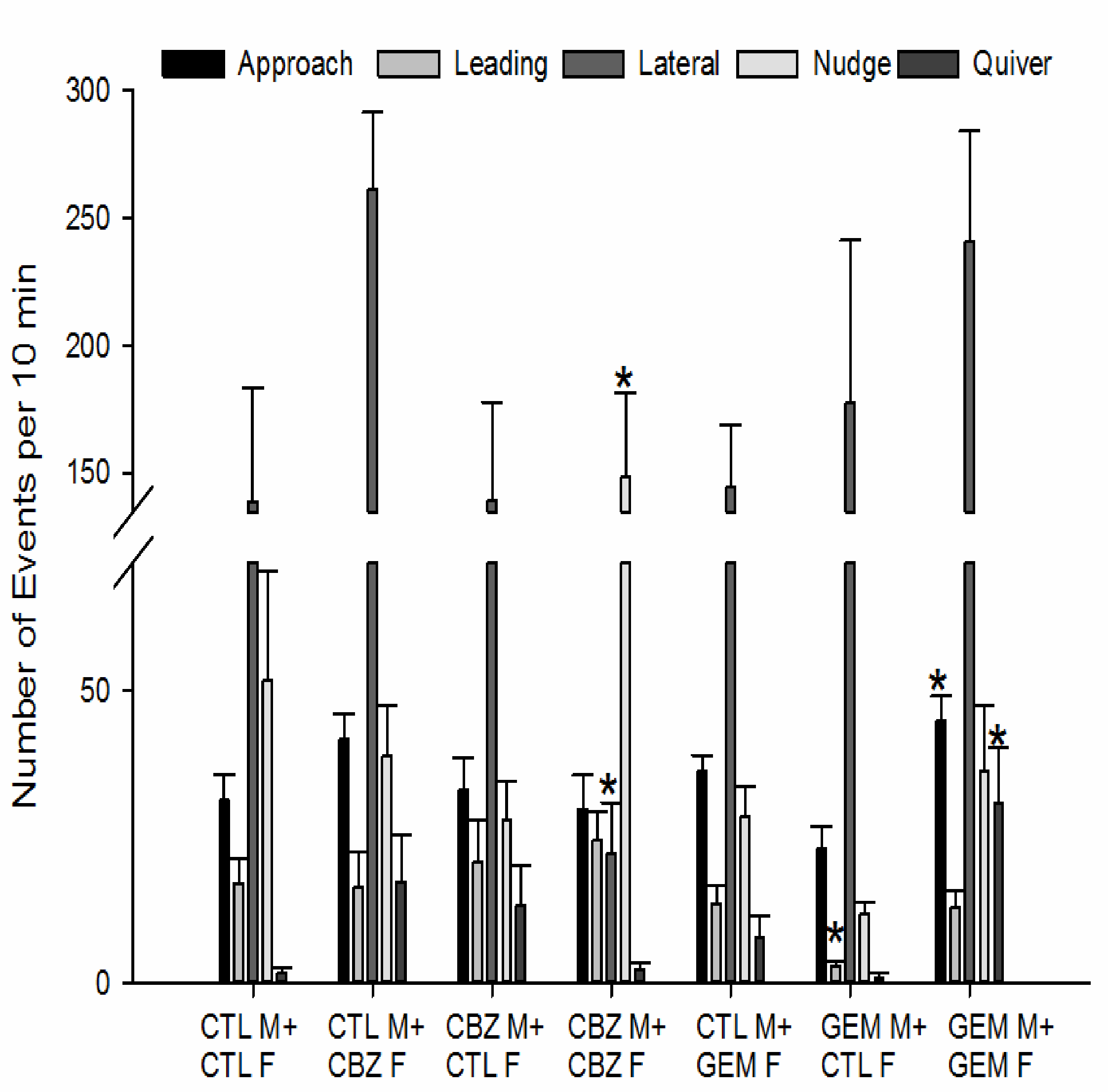

Approach
Leading
Lateral
Nudge
Quiver
Number of Events per 10 min
300
250
200
150
50
0
*
*
*
*
*
CTL M+
CTL F
CTL M+
CBZ F
CBZ M+
CTL F
CBZ M+
CBZ F
CTL M+
GEM F
GEM M+
CTL F
GEM M+
GEM F

**Figure 4.5: Sperm velocity in male $F_1$ zebrafish**. Smooth path velocity ($V_{AP}$) of sperm from $F_1$ males raised in clean water from control (CTL) parents or parents chronically exposed to carbamazepine (CBZ) or gemfibrozil (GEM) for 6 weeks. Sperm was removed from $F_1$ male zebrafish via abdominal massage and expelled on a glass, welled slide. Sperm was then activated with system water (see materials and methods), covered with a coverslip and recorded under 200x magnification using Astro IIDC recording software. $V_{AP}$ was calculated for each male at 4 different 1s intervals; 20, 30,40 and 50s using CEROS computer assisted sperm analysis system. Significant differences were determined using a Mann-Whitney rank sums test. Letters denote signicant differences within each time interval. $p \leq 0.05$. CBZ is carbamazepine, GEM is gemfibrozil, and CTL is control.

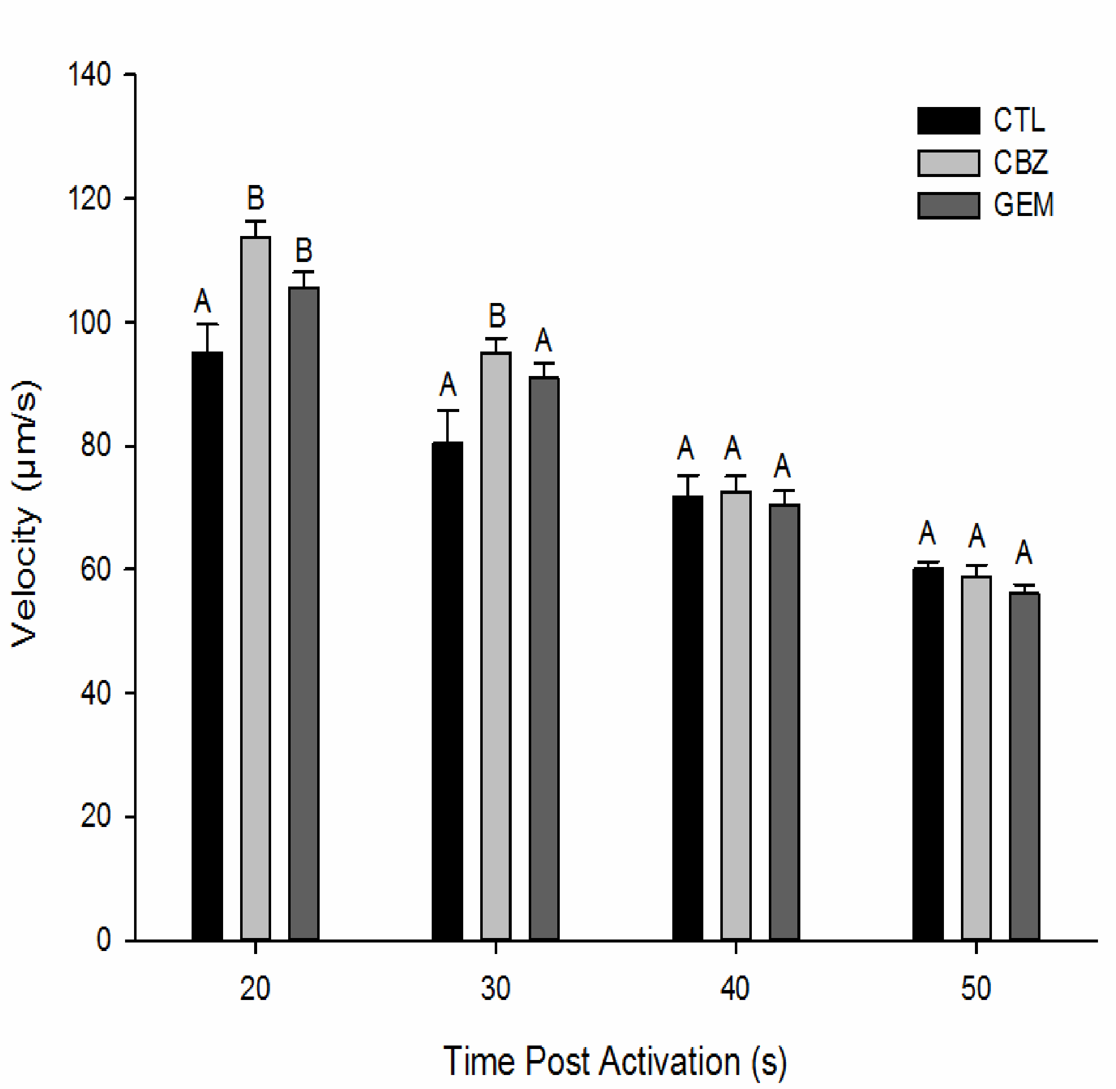
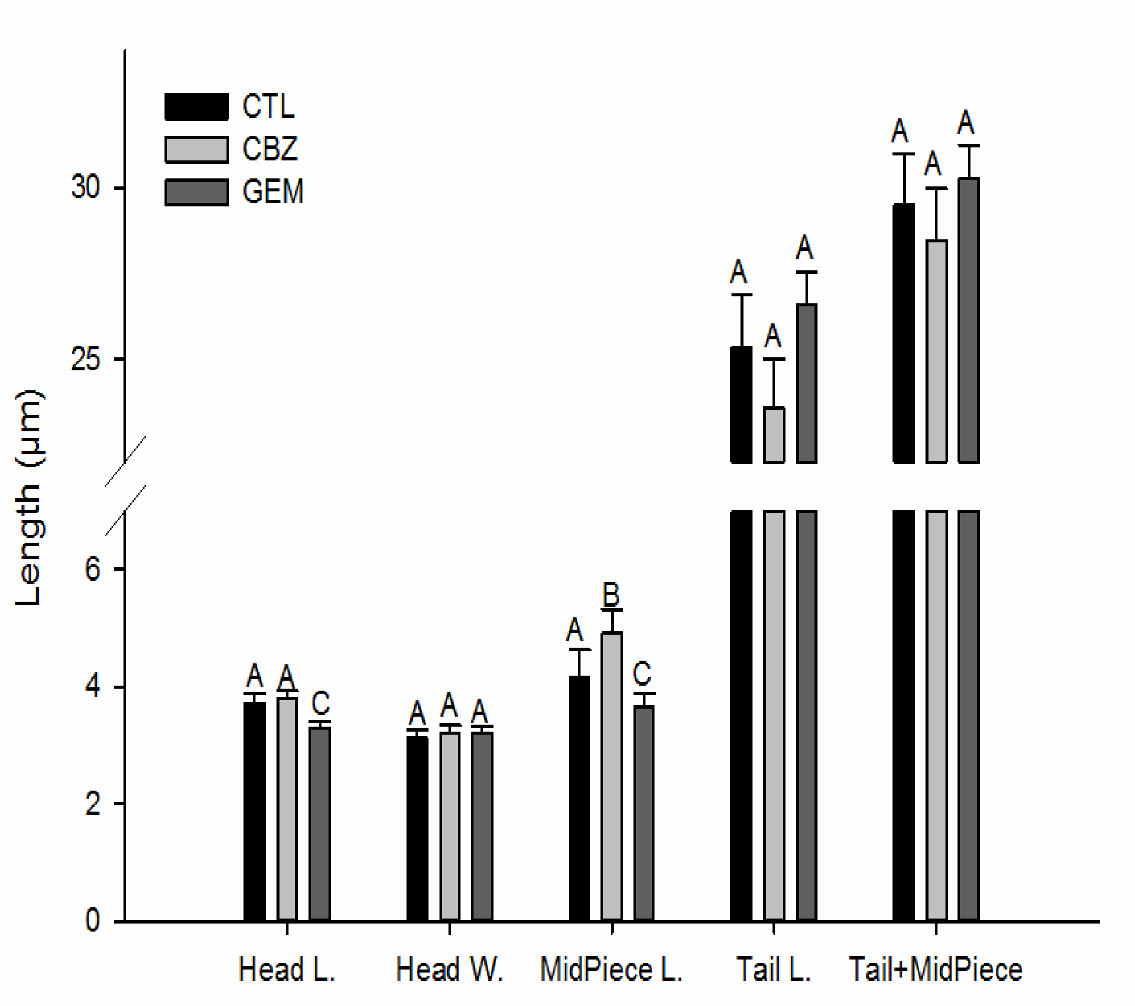

CTL
CBZ
GEM
Velocity (μm/s)
Time Post Activation (s)
0
20
40
60
80
100
120
140
20
30
40
50
A
B
B
A
B
A
A
A
A
A
A
A

**Figure 4.6: Sperm Morphology in male $F_1$ zebrafish**. Sperm head length (L.) and width (W.), midpiece and flagella length of $F_1$ zebrafish males raised in clean water, from control (CTL) parents or parents chronically exposed to carbamazepine (CBZ) or gemfibrozil (GEM) for 6 weeks. Sperm was removed from male $F_1$ zebrafish via abdominal massage and fixed in 10% neutral buffered formalin. 10µl of spermatozoa was diluted 1:1 with distilled water and placed on a glass microscope slide and screened at 400x magnification using a Prosilica EC-650 digital camera mounted on a Leica DMBL microscope and analyzed under phase contrast. Images of 25 spermatozoa per male were taken and measured to the nearest 0.1 µm using ImageJ. Significant differences were determined using a Mann-Whitney rank sums test. Letters denote significant differences with each time interval. $p \leq 0.05$. CBZ is carbamazepine, GEM is gemfibrozil, and CTL is control.


CTL
CBZ
GEM
Length (µm)
0
2
4
6
25
30
Head L.
Head W.
MidPiece L.
Tail L.
Tail+MidPiece
A
A
C
A
A
A
A
B
C
A
A
A
A
A
A

## REFERENCES

Abrams, E.W., Mullins, M.C., 2009. Early zebrafish development: it's in the maternal genes. Current Opinion Gen & Dev 19, 396-403.

Andreozzi, R., Raffaele, M., Nicklas, P., 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere 50, 1319-1330.

Bagenal, T.B., 1969. The Relationship Between Food Supply and Fecundity in Brown Trout Salmo trutta L. J of Fish Biol 1, 167-182.

Bell, A.M., 2001. Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined stickleback, (*Gasterosteus aculeatus*). Animal Behav 62, 775-780.

Benoit, D.A., 1982. User‘s guide for conducting life-cycle chronic toxicity tests with fathead minnows (*Pimephales promelas*). Envrion Research Labs – Duluth, MN. EPA 600/8-81-011.

Bernet, D., Schmidt, H., Meier, W., Burkhardt-Holm, P., Wahli, T., 1999. Histopathology in fish: proposal for a protocol to assess aquatic pollution. J of Fish Diseases 22, 25-34.

Bjerselius, R., Lundstedt-Enkel, K., Olsén, H., Mayer, I., Dimberg, K., 2001. Male goldfish reproductive behaviour and physiology are severely affected by exogenous exposure to 17β-estradiol. Aquat Toxicol 53, 139-152.

Breder, C.M., Rosen, D.E., 1966. Modes of reproduction in fishes. American Museum of Natural History.

Brennan, S.J., Brougham, C.A., Roche, J.J., Fogarty, A.M., 2006. Multi-generational effects of four selected environmental oestrogens on Daphnia magna. Chemosphere 64, 49-55.

Brodie, M.J., Richens, A., Yuen, A.W.C., Group, U.K.L.C.M.T., 1995. Double-blind comparison of lamotrigine and carbamazepine in newly diagnosed epilepsy. The Lancet 345, 476-479.

Casselman, S.J., Schulte-Hostedde, A.I., Montgomerie, R., 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Canadian J of Fisheries and Aqua Sci 63, 2119-2125.

Cole, K., Smith, R.J., 1987. Male courting behaviour in the fathead minnow, *Pimephales promelas*. Environ Biol Fish 18, 235-239.

Csoka, A.B., Szyf, M., 2009. Epigenetic side-effects of common pharmaceuticals: A potential new field in medicine and pharmacology. Medical Hypotheses 73, 770-780.

Darrow, K.O., Harris, W.A., 2004. Characterization and development of courtship in zebrafish, Danio rerio. Zebrafish 1, 40-45.

Devaux, A., Fiat, L., Gillet, C., Bony, S., 2011. Reproduction impairment following paternal genotoxin exposure in brown trout (S*almo trutta*) and Arctic charr (*Salvelinus alpinus*). Aquat Toxicol 101, 405-411.

Dietrich, S., Ploessl, F., Bracher, F., Laforsch, C., 2010. Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in Daphnia magna – A multigenerational study. Chemosphere 79, 60-66.

Dzialowski, E., Turner, P., Brooks, B., 2006. Physiological and Reproductive Effects of Beta Adrenergic Receptor Antagonists in Daphnia magna. Arch Environ Contam Toxicol 50, 503-510.

Eaton, R.C., Farley, R.D., 1974. Spawning cycle and egg production of zebrafish, Brachydanio rerio, in the laboratory. Copeia, 195-204.

EPA, L.-C.A., 1993. Inventory Guidelines and Principles. Risk Reduction Engineering Laboratory, Office of Research and Development, United States Environmental Protection Agency. EPA/600/R-92/245.

Fisk, A.T., Johnston, T.A., 1998. Maternal Transfer of Organochlorines to Eggs of Walleye (Stizostedion vitreum) in Lake Manitoba and Western Lake Superior. J of Great Lakes Research 24, 917-928.

Gage, M.J.G., Macfarlane, C., Yeates, S., Shackleton, R., Parker, G.A., 2002. Relationships between sperm morphometry and sperm motility in the Atlantic salmon. J of Fish Biol 61, 1528-1539.

Galus, M., Jeyaranjaan, J., Smith, E., Li, H., Metcalfe, C., Wilson, J.Y., 2013a. Chronic effects of exposure to a pharmaceutical mixture and municipal wastewater in zebrafish. Aquat Toxicol 132-133, 212-222.

Galus, M., Kirischian, N., Higgins, S., Purdy, J., Chow, J., Rangaranjan, S., Li, H., Metcalfe, C., Wilson, J.Y., 2013b. Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. Aquat Toxicol 132-133, 200-211.

Gerlach, G., 2006. Pheromonal regulation of reproductive success in female zebrafish: female suppression and male enhancement. Animal Behav 72, 1119-1124.

Goetz, F., Garczynski, M., 1997. The ovarian regulation of ovulation in teleost fish. Fish Physiol and Biochem 17, 33-38.

Gonzalez, F.J., 1997. The role of peroxisome proliferator activated receptor alpha in peroxisome proliferation, physiological homeostasis, and chemical carcinogenesis. Adv Exp Med Biol 422, 109-125.

Gray, M.A., Teather, K.L., Metcalfe, C.D., 1999. Reproductive success and behavior of Japanese medaka (Oryzias latipes) exposed to 4-tert-octylphenol. Environ Toxicol Chem 18, 2587-2594.

Greytak, S.R., Tarrant, A.M., Nacci, D., Hahn, M.E., Callard, G.V., 2010. Estrogen responses in killifish (Fundulus heteroclitus) from polluted and unpolluted environments are site- and gene-specific. Aquat Toxicol 99, 291-299.

Hontela, A., Rasmussen, J., Audet, C., Chevalier, G., 1992. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. Arch Environ Contam Toxicol 22, 278-283.

Isojärvi, J., Löfgren, E., Juntunen, K., Pakarinen, A., Päivänsalo, M., Rautakorpi, I., Tuomivaara, L., 2004. Effect of epilepsy and antiepileptic drugs on male reproductive health. Neurology 62, 247-253.

Kim, H., Lee, M., Yu, S., Kim, S., 2012. The individual and population effects of tetracycline on Daphnia magna in multigenerational exposure. Ecotoxicology 21, 993-1002.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999−2000: A National Reconnaissance. Environ Sci Technol 36, 1202-1211.

Lawrence, C., 2007. The husbandry of zebrafish (*Danio rerio*): A review. Aquaculture 269, 1-20.

Li, Z.-H., Li, P., Rodina, M., Randak, T., 2010. Effect of human pharmaceutical Carbamazepine on the quality parameters and oxidative stress in common carp (*Cyprinus carpio L.*) spermatozoa. Chemosphere 80, 530-534.

Lüpold, S., Calhim, S., Immler, S., Birkhead, T.R., 2009. Sperm morphology and sperm velocity in passerine birds. Proc Royal Soc B: Biol Sci 276, 1175-1181.

Lürling, M., Sargant, E., Roessink, I., 2006. Life-history consequences for Daphnia pulex exposed to pharmaceutical carbamazepine. Environ Toxicol 21, 172-180.

Malo, A.F., Gomendio, M., Garde, J., Lang-Lenton, B., Soler, A.J., Roldan, E.R., 2006. Sperm design and sperm function. Biol Lett 2, 246-249.

Martin, G., Schoonjans, K., Lefebvre, A.-M., Staels, B., Auwerx, J., 1997. Coordinate Regulation of the Expression of the Fatty Acid Transport Protein and Acyl-CoA Synthetase Genes by PPARα and PPARγ Activators. J of Biological Chem 272, 28210-28217.

McCormick, M.I., 1998. Behaviorally induced maternal stress in a fish influences progeny quality by a hormonal mechanism. Ecology 79, 1873-1883.

Metcalfe, C.D., Miao, X.S., Koenig, B.G., Struger, J., 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ Toxicol and Chem / SETAC 22, 2881-2889.

Mhanni, A.A., McGowan, R.A., 2004. Global changes in genomic methylation levels during early development of the zebrafish embryo. Dev Genes Evol 214, 412-417.

Migliaccio, S., Newbold, R., Bullock, B., Jefferson, W., Sutton, F., McLachlan, J., Korach, K., 1996. Alterations of maternal estrogen levels during gestation affect the skeleton of female offspring. Endocrinology 137, 2118-2125.

Mileva, V.R., Gilmour, K.M., Balshine, S., 2011. Effects of maternal stress on egg characteristics in a cooperatively breeding fish. Compar Biochem Physiol A 158, 22-29.

Mimeault, C., Woodhouse, A.J., Miao, X.S., Metcalfe, C.D., Moon, T.W., Trudeau, V.L., 2005. The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, *Carassius auratus*. Aquat Toxicol 73, 44-54.

Nacci, D.E., Gleason, T.R., Munns, W.R., 2002. Evolutionary and Ecological Effects of Multi-Generational Exposures to Anthropogenic Stressors. Human Ecol Risk Assess 8, 91-97.

Nakari, T., Erkomaa, K., 2003. Effects of phytosterols on zebrafish reproduction in multigeneration test. Environ Poll 123, 267-273.

Nash, J.P., Kime, D.E., Van der Ven, L.T., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R., 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Environ Health Persp 112, 1725.

Nassef, M., Matsumoto, S., Seki, M., Khalil, F., Kang, I.J., Shimasaki, Y., Oshima, Y., Honjo, T., 2010. Acute effects of triclosan, diclofenac and carbamazepine on feeding performance of Japanese medaka fish (*Oryzias latipes*). Chemosphere 80, 1095-1100.

Nye, J.A., Davis, D.D., Miller, T.J., 2007. The effect of maternal exposure to contaminated sediment on the growth and condition of larval Fundulus heteroclitus. Aquat Toxicol 82, 242-250.

O'Brien, M.L., Twaroski, T.P., Cunningham, M.L., Glauert, H.P., Spear, B.T., 2001. Effects of peroxisome proliferators on antioxidant enzymes and antioxidant vitamins in rats and hamsters. J Soc Toxicol 60, 271-278.

OECD, 2002. Fish two-generation test guideline. Draft proposal for a new guideline. Organisation for Economic Cooperation and Development, Paris.

Ortiz-Rodríguez, R., Dao, T.S., Wiegand, C., 2012. Transgenerational effects of microcystin-LR on Daphnia magna. J of of Exp Biol 215, 2795-2805.

Oshima, Y., Kang, I.J., Kobayashi, M., Nakayama, K., Imada, N., Honjo, T., 2003. Suppression of sexual behavior in male Japanese medaka (*Oryzias* latipes) exposed to 17β-estradiol. Chemosphere 50, 429-436.

Pack, A., 2005. Effects of treatment on endocrine function in patients with epilepsy. Current treatment options in neurology 7, 273-280.

Parker, G.A., 1993. Sperm competition games: sperm size and sperm number under adult control. Proceedings. Biolo Sci 253, 245-254.

Parrott, J.L., Bennie, D.T., 2009. Life-cycle exposure of fathead minnows to a mixture of six common pharmaceuticals and triclosan. J of Toxicol and Environ Health . Part A 72, 633-641.

Paxéus, N., 2004. Removal of selected non-steroidal anti-inflammatory drugs (NSAIDs), gemfibrozil, carbamazepine, beta-blockers, trimethoprim and triclosan in conventional wastewater treatment plants in five EU countries and their discharge to the aquatic environment. Water Sci Technol 50, 253-260.

Péry, A.R.R., Gust, M., Vollat, B., Mons, R., Ramil, M., Fink, G., Ternes, T., Garric, J., 2008. Fluoxetine effects assessment on the life cycle of aquatic invertebrates. Chemosphere 73, 300-304.

Petrović, M., Gonzalez, S., Barceló, D., 2003. Analysis and removal of emerging contaminants in wastewater and drinking water. Trends in Anal Chem 22, 685-696.

Potts, G.W., 1984. Parental behaviour in temperate marine teleosts with special reference to the development of nest structures. Fish Repro, 223-244.

Rao, J.S., Lee, H.J., Rapoport, S.I., Bazinet, R.P., 2008. Mode of action of mood stabilizers: is the arachidonic acid cascade a common target? Molecular Psychiatry 13, 585-596.

Ruiz-Pesini, E., Diez, C., Lapeña, A.C., Pérez-Martos, A., Montoya, J., Alvarez, E., Arenas, J., López-Pérez, M.J., 1998. Correlation of sperm motility with mitochondrial enzymatic activities. Clinical Chem 44, 1616-1620.

Runnalls, T.J., Hala, D.N., Sumpter, J.P., 2007. Preliminary studies into the effects of the human pharmaceutical Clofibric acid on sperm parameters in adult Fathead minnow. Aquat Toxicol 84, 111-118.

Russell, R.W., Gobas, F.A.P.C., Haffner, G.D., 1998. Maternal Transfer and in Ovo Exposure of Organochlorines in Oviparous Organisms: A Model and Field Verification. Environ Sci Technol 33, 416-420.

Schulz, R., Vischer, H., Cavaco, J., Santos, E., Tyler, C., Goos, H., Bogerd, J., 2001. Gonadotropins, their receptors, and the regulation of testicular functions in fish. Compar Biochem and Physiol Part B: 129, 407-417.

Snook, R.R., 2005. Sperm in competition: not playing by the numbers. Trends in Ecolo & Evo 20, 46-53.

Sorensen, P., Brash, A., Goetz, F., Kellner, R., Bowdin, L., Vrieze, L., 1995. Origins and functions of F prostaglandins as hormones and pheromones in the goldfish, Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish. Austin Texas, FishSymp, pp. 252-254.

Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2008. The behaviour and ecology of the zebrafish, *(Danio rerio*). Biol rev Cam Philos Soc 83, 13-34.

Spencer, C.M., Barradell, L.B., 1996. Gemfibrozil. A reappraisal of its pharmacological properties and place in the management of dyslipidaemia. Drugs 51, 982-1018.

Warner, R.R., 1997. Sperm Allocation in Coral Reef Fishes. BioScience 47, 561-564.

Westerlund, L., Billsson, K., Andersson, P.L., Tysklind, M., Olsson, P.-E., 2000. Early life-stage mortality in zebrafish (*Danio rerio*) following maternal exposure to polychlorinated biphenyls and estrogen. Environ Toxicol Chem 19, 1582-1588.

Zhang, X., Oakes, K.D., Cui, S., Bragg, L., Servos, M.R., Pawliszyn, J., 2010. Tissue-Specific In Vivo Bioconcentration of Pharmaceuticals in Rainbow Trout (*Oncorhynchus mykiss*) Using Space-Resolved Solid-Phase Microextraction. Environ Sci Technol 44, 3417-3422.

Zhang, Y., Geißen, S.-U., Gal, C., 2008. Carbamazepine and diclofenac: Removal in wastewater treatment plants and occurrence in water bodies. Chemosphere 73, 1151-1161.

# CHAPTER 5

## PROSTAGLANDINS PREVENT ACETAMINOPHEN INDUCED EMBRYO TOXICITY IN ZEBRAFISH (*Danio rerio*)

Submitted to the American Association of Pharmaceutical Scientists Journal.
[Galus, M., Gugilla, A, Jönsson, M., Wilson, J.Y., 2014. AAPSJ. S-14-00216] © 2014

## ABSTRACT

Previous research in our laboratory has shown that acetaminophen (ACE) induced embryonic mortality and abnormalities in zebrafish at 0.5 and 10 ug $L^{-1}$. In this study, we have examined the dose response relationships between aqueous ACE exposure (0.05-50 µg $L^{-1}$) and mortality or developmental abnormalities in developing zebrafish. The dose response relationship for total abnormality showed that concentrations as low as 0.1 µg $L^{-1}$ significantly increased the abnormality rate. All concentrations of ACE significantly increased mortality rate compared to controls. In mammals, ACE decreases prostaglandin production through the inhibition of cyclooxygenase (COX) enzymes. Here we report COX activity in zebrafish embryos throughout development and the expression patterns of the *cox*-1, *cox*-2a, and *cox*-2b genes. Embryonic COX activity was significantly inhibited by specific mammalian *cox*-1 (SC-560) and *cox*-2 (DuP-697) inhibitors in unexposed and ACE-exposed fish. COX activity declined with development time. Expression of *cox*-1, *cox*-2a and *cox*-2b were found at 1 hour post fertilization, indicating maternal deposition of the transcripts. Embryonic expression of all COX genes began in gastrulation or early segmentation. Co-exposure to ACE (0.5 µg $L^{-1}$) and prostaglandin E2 at 0.1-1 µM abolished the ACE-induced mortality and abnormalities. This strongly supports the hypothesis that ACE-induced mortalities and abnormalities in zebrafish embryos were mediated by inhibition of COX activity and reduced prostaglandin levels. This research demonstrates that acetaminophen, and likely other COX inhibitors, elicit their embryo toxicity in fish though a molecular mechanism of action that is identical to their therapeutic effects in mammals.

## INTRODUCTION

The role of cyclooxygenase (COX) enzymes is to catalyze the conversion of arachidonic acid (AA) to prostaglandin H2 (PGH2). PGH2 is then further isomerized by a suite of tissue specific synthases to common precursors of various prostanoids, which include other prostaglandins, prostacyclins, and thromboxanes (Simmons et al., 2004; Vane et al., 1998). In mammals, prostaglandins play important roles in a wide range of biological processes throughout all stages of life. They are involved in platelet aggregation, glomerular and hemostatic homeostasis, antipyretic and analgesic control and regulating inflammatory responses (Buck et al., 1981; Gresele et al., 1987). In fish species (Cha et al., 2005; Grosser et al., 2002; Lister and Van Der Kraak, 2008), and similar to mammals (Elvin et al., 2000; Eppig, 2001; Gilchrist et al., 2004), prostaglandins have known roles in reproduction, oocyte maturation and germ line development. Our understanding of the cyclooxygenase-prostaglandin system has been largely due to the advances made with mammalian models. However, recent work amongst invertebrates (Rowley et al., 2005) has demonstrated that prostaglandins are involved in reproduction and ion regulation suggesting that the function of these bioactive lipids are likely evolutionarily conserved outside of vertebrates.

Mammalian species have two cyclooxygenase genes, cyclooxygenase 1 (*cox*-1) (Yokoyama et al., 1988) and cyclooxygenase 2 (*cox*-2) (Rosen et al., 1989). *cox*-1 is the isoform which is constitutively expressed in many tissues and often referred to as the housekeeping gene responsible for basal prostaglandin production (Yasojima et al., 1999).

*cox*-2 is not constitutively expressed and only found in select cell types where it is inducible by cytokines, mitogens or growth factors (Yasojima et al., 1999). The mammalian *cox*-1 and *cox*-2 proteins are homologs with 77% similar amino acid sequence and 63% similar crystal structure (Gierse et al., 1996).

Cyclooxygenase orthologs have been identified in many teleosts including rainbow trout (*Oncorhynchus mykiss*) (Zou et al., 1999), brook trout (*Salvelinus fontinalis*) (Roberts et al., 2000) and zebrafish (*Danio rerio*) (Grosser et al., 2002). The amino acid sequence similarity of fish COX enzymes to their mammalian orthologs range between 65 and 73% (Grosser et al., 2002). As in mammals, *cox*-1 is constitutively expressed and responsible for basal prostaglandin production (Grosser et al., 2002) while *cox*-2 is expressed only in certain cell types and is sensitive to induction by cytokines, mitogens and growth factors (Ishikawa et al., 2007). Unique to zebrafish and rainbow trout is a duplication of the *cox*-2 genes, *cox*-2a and *cox*-2b; *cox*-2b has been identified and characterized as a paralog to zebrafish *cox*-2a (Ishikawa et al., 2007). Very little is known about *cox*-2b but it appears to share a common expression pattern and inducibility with mammalian *cox*-2 (Ishikawa et al., 2007). The literature typically uses *cox*-2 as synonymous to *cox*-2a, however in this manuscript we will distinguish between the paralogs and only use *cox*-2 to describe orthologs not in zebrafish.

COX genes in fish are involved in many physiological processes. However, they are particularly active during early fish embryogenesis, as prostaglandins are important during key developmental periods such as gastrulation and segmentation (Grosser et al., 2002; Ishikawa et al., 2007; Prescott and Yost, 2002). In zebrafish, *cox*-1 was shown to

play a critical role during gastrulation (5-10 hpf), a period of rapid cell proliferation and migration; thus prostaglandins (in particular PGE2) are thought to be involved in activating cells to migrate to their correct embryonic germ layers (Cha et al., 2006b; Grosser et al., 2002; Ishikawa et al., 2007). *cox*-2a was active during segmentation (11-24 hpf); suggesting a role for prostaglandins in posterior mesoderm development and isolation of various body segments (Grosser et al., 2002; Ishikawa et al., 2007; Jönsson et al., 2012).

The knockdown of COX gene expression during zebrafish embryogenesis can lead to the onset of developmental abnormalities. *cox*-1 knockdown using antisense morpholino oligonucleotides (10ng/embryo) resulted in gastrulation arrest and defects in vascular tube structures (Cha et al., 2006b; Grosser et al., 2002). However, knockdown of *cox*-2b showed no alterations in phenotype; suggesting that as in mammals, *cox*-1 plays a larger role during development than *cox*-2b (Cha et al., 2006b; Grosser et al., 2002); the potential role of *cox*-2b in development is not yet clear.

Pharmacological inhibition of COX enzymes is possible through the use of non-steroidal anti-inflammatory drugs (NSAIDs) like acetaminophen (ACE), a frequently used over-the-counter analgesic and anti-inflammatory drug. ACE has been consistently detected in the aquatic environment at or above concentrations of 0.1 μg $L^{-1}$ (Andreozzi et al., 2003; Brun et al., 2006; Kolpin et al., 2002). There have been several studies documenting adverse effects of NSAID exposure in fish at low concentrations. Larval zebrafish exposed to ibuprofen (10 - 100 μg $L^{-1}$) or ACE (0.5 - 100 μg $L^{-1}$) have shown several developmental effects including decreased hatching and growth rates, increased rates of spinal deformation and increased mortality (David and Pancharatna, 2009; Galus

et al., 2013; Weigt et al., 2010). COX inhibition and decreased prostaglandin production is the presumed mechanism by which these compounds are thought to produce embryo toxicity fish. Yet, this mechanism has not been clearly demonstrated especially at the low concentrations of exposures reported.

In this study, we further elucidate the impacts of ACE on cyclooxygenase inhibition and embryo toxicity in zebrafish. We better characterize the toxicity of ACE during developmental processes in zebrafish by examining dose response relationships, time course for effects, and the expression and activity of COX genes during development. We have chronically exposed zebrafish to aqueous ACE and monitored effects throughout the course of development. Exposed embryos were co-incubated with synthetic prostaglandin E2 (PGE2) in attempts to reduce the abnormalities or mortality observed with ACE exposure alone. These data are important to help better understand the mechanistic action of acetaminophen in fish development.

## METHODS

### *Fish Care*

Wild-type, adult zebrafish (AQUAlity Tropical Fish Wholesale, Canada) were housed at 4 fish $L^{-1}$ and 1:1 sex ratio in a recirculating system (28°C, pH 7-8, dissolved oxygen ≥87% and conductivity 470-455 μS) with ≥10% daily renewal. Aquaria water was made from distilled water supplemented with sodium bicarbonate and sea salts (Instant Ocean, Spectrum Brands, USA) to achieve the conditions above. Fish were fed twice with a commercial food (Tetramin Tropical Flakes, Tetra, USA) and once with live, adult Artemia (GSL Brine Shrimp, USA) each day. Zebrafish were kept on a 14:10 hour

(light:dark) light cycle. Fish were maintained in our facility for a minimum of one month prior to use in any experiment and breeding was monitored on a weekly basis. All animal holding, breeding, and experimentation were performed in accordance to McMaster University's animal care policies and under an approved animal use protocol.

*Compounds Tested*

All pharmacological agents were purchased from Sigma Aldrich (Sigma Aldrich, Canada), except 6-formylindolo[3,2-b]carbazole (FICZ) which was purchased from Syntastic AB (Stockholm, Sweden). Stock solutions of ACE where made at 2 mg $mL^{-1}$ and directly dissolved into E3 embryo rearing media (1 mL $well^{-1}$; NaCl 5 μg $L^{-1}$, KCl 0.17 μg $L^{-1}$, CaCl2 0.33 μg $L^{-1}$ and MgSO4 0.33 μg $L^{-1}$). A stock of PGE2 was made at 1 mg $mL^{-1}$ in distilled water and diluted with E3 media to produce appropriate working solutions. Exposure solutions were renewed every 24 hr. Nominal concentrations of ACE were not verified because of the small volumes used in each well but compound preparation was identical to a prior study and the concentrations and stability of ACE with this experimental design have previously been published (Galus et al., 2013).

*Experimental Design*

Fertilized embryos from unexposed parents were collected 0.5 hrs after first light (1-2 cell stage) from holding tanks, which comprised of 50 adult zebrafish at a 1:1 sex ratio. Embryos from all tanks were pooled in petri plates containing E3 media, counted and equally divided into separate petri plates at 50 embryos per plate containing 10 mL of exposure solution ensuring that all embryos were dosed at the same time. Embryos were transferred to individual wells in 48 well plates containing 1 mL $well^{-1}$ aliquots of exposure

solution. At least one 48 well control plate was followed for each embryo collection to determine background mortality for each embryo collection. Mortality can be variable across clutches of embryos, however we typically observe less than 10% mortality amongst embryos collected from unexposed parents. If mortality exceeded 10% within controls, all exposure plates from the same embryo collection were discarded. Replicate plates were completed with different clutches.

*Acetaminophen Dose Response*

Zebrafish embryos were exposed to 0 (CTL) and 0.05, 0.1, 0.5, 1, 5, 10, 20 and 50 µg $L^{-1}$ of ACE. Embryos were incubated at 28.5°C in 48 well plates and observed at 6, 24, 48 and 72 (hpf) for the rate of abnormality (yolk sac or pericardial edema, stunted growth or spinal deformation) and mortality. Exposure solutions were renewed every 24 hrs. N=8 (CTL) and N=5 plates for each concentration of ACE.

*Acetaminophen Time Course*

Zebrafish embryos were exposed to 0 (CTL) and 0.5 µg $L^{-1}$ of ACE. Embryos were incubated at 28.5°C in 48 well plates and observed at 3, 6, 9, 12, 15, 20, 24, 48 and 72 hpf for the rate and timing of mortality, abnormality and hatching success. N=7 plates for CTL and ACE treatment groups.

*Total COX Activity*

Total embryonic COX activity was determined using COX Activity Assay with slight modifications to the manufacturer's instructions (Kit #760151; Cayman Chemical, Ann Arbor, MI, USA). Briefly, CTL (0) and ACE (0.5 µg $L^{-1}$) exposed embryos were

collected in pools at 0, 3, 6, 9, 12, 15, 20, 24, 48 and 72 hpf and flash frozen in liquid nitrogen. Embryos (N=100 per pool) were extracted in 1000 µL of 100 mM Tris HCL (pH 7.5) spiked with 0.1 mM phenylmethanesulfonyl fluoride. For each sample, total COX activity and total COX activity in the presence of the mammalian COX inhibitors SC – 560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl-1H-pyrazole; *cox*-1 inhibitor; 66 µM) or DuP – 697 (5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonyl)phenyl-thiophene; *cox*-2 inhibitor; 60 µM) was measured. COX activity is reported in nmol/min/ml as reported in a prior studies (Bivol et al., 2008; Toller et al., 2010). All samples were run in duplicate and N=3 pools of embryos for each treatment.

*COX Gene Expression*

Groups of 90 zebrafish embryos (AB type) were placed in each of four large glass petri dishes (diameter: 14 cm) containing 90 ml of carbon-filtered Uppsala tap water. At 2.5 hpf DMSO was added to two dishes and while FICZ dissolved in DMSO was added to the other two dishes yielding final nominal concentrations of 100 ppm DMSO and 10 nM FICZ. The dishes were incubated at 28.5°C. One sample of 10 pooled embryos was collected from each dish at 3, 5, 7, 9, 11, 13, 15, and 26 hpf (n=2), and immediately flash frozen in liquid nitrogen. Duplicate samples of 10 pooled unexposed embryos at the 2-4 and 8-16 cell stages (approximately 1 and 1.5 hpf) were collected and frozen liquid nitrogen. The samples were stored at -80°C until used for quantitative real time RT-PCR (qPCR) analysis.

RNA extraction, cDNA synthesis, and qPCR analysis were performed as described by (Gao et al., 2011). Total RNA was isolated and DNAse-treated using the Aurum™ Total

RNA Fatty and Fibrous Tissue kit (Bio-Rad Laboratories Inc., Hercules CA, USA) according to the instructions. The concentration of RNA was determined spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Total RNA, 0.5 μg per reaction, was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad). Gene-specific real time PCR primers for *cox*-1, *cox*-2a, and *cox2*-b (GenBank: NM_153656.1, NM_153657.1, and NM_001025504.2) were synthesized by Sigma-Aldrich. New primers were designed for *cox*-1 with the following sequences (5′to 3′): F-TGAAGTACCAGGTGCTCAACG and R-GCTCTGGAGG GACTGATG. The *cox*-2 primer sequences are previously published: *cox*-2a F-ACTACCCCTGAGCTTCTCACA and R-GATGCTGTTGATGATATCCCAGATTG and *cox*-2b F- ATTGGTGAGACTAT and R-TCGGGATCAAACTTGAG CTTAAAATA (Jönsson et al., 2012). Reaction solutions (20 μL) were composed by mixing cDNA (12.5 ng), forward and reverse primers (5 pmol of each), water, and iQ SYBR Green Supermix (Bio-Rad). Real time PCR was performed with a Rotor Gene 6000 (Qiagen, Hilden, Germany). Samples were analyzed in duplicate with the following protocol: 95 °C for 3 min, and then 40 cycles of 95 °C for 15 s and 62 °C for 45 s. To ensure that a single product was amplified, melt curve analysis was performed on the PCR products at the end of each PCR run.

Because reference transcripts and other transcripts can show a considerable variation in expression over development generally, normalization to a reference gene is likely to distort the results. Therefore levels of mRNA expression were calculated without normalization, i.e., by E−CT (Schmittgen and Livak, 2008). For each primer pair PCR

efficiencies (E) were determined by the LinRegPCR program (Ruijter et al., 2009). Data are presented as mean+SEM.

*Prostaglandin E2 Supplementation*

Zebrafish embryos were exposed to 0 (CTL), 0.5 µg $L^{-1}$ of ACE alone or 0.5 µg $L^{-1}$ of ACE co-exposed with 0.01, 0.1, 0.5 or 1 µM PGE2. Embryos were incubated at 28.5°C in 48 well plates and observed at 3, 6, 9, 12, 15, 20, 24, 48 and 72 hpf for the rate and timing of mortality, abnormality and hatching success. Embryos number equals N=48 (CTL), N=48 (ACE alone) and N=48 for each dose PGE2 in the ACE+PGE2 treatments.

*Statistics*

All statistical tests were analyzed using SigmaPlot 11.0 (Systat Software). Normality of the data was determined using the Shapiro-Wilk's test. The dose response relationships for abnormality and mortality were analyzed using a one-way analysis of variance (ANOVA) comparing individual doses back to CTL. The incidence of developmental abnormalities and mortality within larval time course exposures was analyzed using individual t-tests comparing CTL to ACE exposed embryos at each time point. Mortality, hatching, and developmental abnormalities were normalized to the number of embryos at 72 hpf. Total COX activity data within CTLs was analyzed using a one-way ANOVA with comparison to CTL. Differences between CTL vs ACE, CTL vs blockers and ACE vs blockers were analyzed using a t-test at each time point. Abnormality and mortality rates in the prostaglandin supplementation experiment were analyzed using a chi-square test comparing exposed animals to CTL. A Holm-Sidak post-hoc test was used

to determined significant differences after ANOVA analysis. All data was expressed as mean±SEM and the significance level was set at $P \leq 0.05$.

**RESULTS**

*Acetaminophen Dose Response Curve*

The dose response relationship amongst embryonic zebrafish exposed to 0.05 - 50 μg $L^{-1}$ of ACE shows that doses at 0.1 μg $L^{-1}$ or higher elicit levels of abnormality that are different from controls (Figure 5.1). The maximal effect was observed at concentrations > 5 μg $L^{-1}$ (Figure 5.1). Abnormalities typically observed within the exposed population consisted of yolk sac or pericardial edema, stunted growth or spinal deformation. The dose response relationship for mortality shows peak mortality observed at the 1 μg $L^{-1}$ with all doses significantly increasing mortality rate over CTLs; yet the data is quite variable between doses (Figure 5.2).

*Acetaminophen Time Course*

Embryonic zebrafish were exposed to ACE at 0.5 ug $L^{-1}$ and examined at 3, 6, 9, 12, 15, 20, 24, 36, 48 and 72 hpf for abnormalities (Figure 5.3), mortality (Figure 5.4) and hatching success (data not shown). Detailed screening of larval fish was conducted during gastrulation (5 – 10 hpf) and segmentation (11 – 24 hpf) because our prior study found ACE induced higher mortality by 24 hpf than later in development (Galus et al., 2013). Total abnormalities in ACE exposed fish peaked with a 2.5-3 fold increase over control animals at 6, 9 and 15 hpf (Figure 5.3). Abnormality rate remained elevated over control animals throughout gastrulation and segmentation and dropped below CTL animals at 24 hpf (Figure 5.3). At the early time points, the abnormality was edema in the blastula which

often progressed into pericardial or yolk sac edema at later time points, if the embryos survived.

The time course for mortality was similar to abnormalities. Mortality was 3.1 fold over CTL animals during gastrulation (Figure 5.4). Mortality amongst CTL animals was not observed between 15 and 24 hpf but remained elevated amongst ACE exposed animals (Figure 5.4). Mortality was mainly observed in those animals that developed abnormalities prior to 24 hpf. Although the time specific abnormality and mortality rates observed throughout gastrulation and segmentation were not statistically different, total abnormality and mortality rates were similar to rates in Figure 5.1 and 5.2 and statistically different from controls. There was no significant impact on hatching rate as greater than 99% of embryos hatched by 48 hpf.

*Total COX Activity*

Total COX activity was measured in embryonic zebrafish throughout development (Figure 5.5, Table 5.1). Within CTL animals, there was no significant difference in COX activity between 3 and 24 hpf; COX activity was significantly lower at 48 and 72 hpf compared to animals at 3 and 6 hpf (Table 5.1). Embryos exposed to 0.5 ug $L^{-1}$ ACE showed similar levels of COX activity for most time points (Figure 5.5). COX activity was significantly elevated with ACE exposure at 3 hpf compared to CTL and significantly lower with ACE exposure at 15 and 20 hpf compared to CTL animals (Figure 5.5). COX activity was significantly reduced from 42-83% with the addition of potent mammalian *cox*-1 and *cox*-2 inhibitors, SC-560 and DuP-697 (Figure 5.5B). Samples treated with SC-560 showed, on average, 80% inhibition. DuP-697 treated samples were inhibited by at least 42

– 63% with the exception of the 48 hpf ACE treated sample which was inhibited 83% by DuP-697 (Figure 5.5B). All inhibitor treated samples showed significant reductions in COX activity except CTL samples at 9 and 12 hpf, which showed no inhibition of COX activity with SC-560 (Figure 5.5B), likely due to high variation in the samples with inhibitor.

*Gene Expression*

Levels of *cox*-1, 2a and 2b mRNA were determined in embryonic zebrafish during the first day of development. Transcript levels were measured in unexposed embryos two time points where cell stages were ≤16 cells (1 and 1.5 hpf) and in embryos exposed to DMSO or 10 nM FICZ between 2.5 (blastula period, >128 cells) and 26 (pharyngula period, Prim-5-15) hpf. All three COX genes were detected at 1 and 1.5 hpf. The levels of *cox*-1 mRNA remained similar during the first 9 h after fertilization but showed an increase between 11 and 15 hpf (Figure 5.6A). Expression of *cox*-2a remained similar until 7 hpf when the transcript levels increased and remained elevated (Figure 5.6B). The transcript levels of *cox*-2b were mildly increased at 7 and 9 hpf and subsequently deceased (Figure 5.6C). Hence, expression of *cox*-2b was higher during gastrulation (5-10 hpf), while *cox*-1 expression peaked during early segmentation (13 hpf), and *cox*-2a expression was higher during both of these periods. We hypothesized that AhR could play a role in regulating COX gene expression because there are reports of upregulation of *cox*-2 through AhR activation (Martey et al., 2005). Thus, we examined the expression of zebrafish COX genes with exposure to FICZ, a tryptophan derived photoproduct with high affinity for the AhR. Exposure to FICZ had no effect on the time course of any of the three transcripts.

*Prostaglandin E2 Supplementation*

Zebrafish embryos were co-exposed to PGE2 and 0.5 μg $L^{-1}$ ACE. Similar to the time course experiment, exposure at 0.5 μg $L^{-1}$ of ACE alone caused a significant increase in abnormalities (Figure 5.7) and mortality (Figure 5.8) over control animals. Co-exposure of ACE with 0.1, 0.5 or 1 μM PGE2 significantly reduced abnormalities and mortality to those found in CTLs. PGE2 co-exposure at 0.01 μM did not reduce abnormalities or mortality compared to 0.5 μg $L^{-1}$ of ACE alone.

**DISCUSSION**

The aim of this study was to determine the mechanism of action of ACE in developmental toxicity within a fish model. For this purpose, we have exposed embryonic zebrafish to ACE and monitored survival and development of exposed embryos. We quantified cyclooxygenase activity and gene expression and co-exposed animals to ACE and exogenous synthetic prostaglandin. Zebrafish were most sensitive to ACE during gastrulation and segmentation, developmental periods which are dependent on cyclooxygenase pathways (Cha et al., 2005; Cha et al., 2006b; Grosser et al., 2002) and had measureable COX activity and gene expression. This, coupled with the rescue of ACE induced toxicity with exogenous PGE2 provides strong evidence that acetaminophen is eliciting its developmental toxicity in zebrafish via the cyclooxygenase pathway. We propose that the toxicity of low concentration non-steroidal anti-inflammatory drugs in embryonic fish is mediated by reduced prostaglandin synthesis during critical developmental processes.

*Acetaminophen Dose Response*

Embryonic ACE exposure at 50 and 100 µg $L^{-1}$ reduced responses to sound, light and touch stimuli in newly hatched fry (David and Pancharatna, 2009) and lead to cellular apoptosis within the developing tail bud (Parng et al., 2004). We have previously exposed embryonic zebrafish to 0.5 and 10 µg $L^{-1}$ ACE and observed elevated rates of mortality and abnormality (Galus et al., 2013). Effects were significantly larger in the 0.5 µg $L^{-1}$ compared to the 10 µg $L^{-1}$ exposure group (Galus et al., 2013). Dose response relationships determined over 0.05 – 50 µg $L^{-1}$ ACE showed that embryos exposed to concentrations of acetaminophen as low as 0.1 µg $L^{-1}$ had elevated rates of developmental abnormalities (Figure 5.1). The fact that such small concentrations are capable of causing toxicity is important given that environmentally relevant concentrations of acetaminophen are reported between 0.1 and 0.5 µg $L^{-1}$ (Andreozzi et al., 2003; Brun et al., 2006; Kolpin et al., 2002). Exposures between 1 and 10 µg $L^{-1}$ ACE sometimes resulted in animals with multiple abnormalities, such as yolk and pericardial edema and spinal deformation, something not seen in the controls (data not shown). Interestingly, mortality (Figure 5.2) was elevated at concentrations as low as 0.05 µg $L^{-1}$, suggesting that mortality is one of the most sensitive endpoints reported for developmental ACE exposures. Surprisingly, the dose response curve did not show a peak median dose, as our prior experiments had suggested (Galus et al., 2013). Indeed, there was no significant difference between 0.5 and 10 µg $L^{-1}$ as we had previously reported (Galus et al., 2013). The reasons for this discrepancy are not yet clear but the variation in mortality and abnormality rate is fairly large suggesting significant differences in inter-clutch responsiveness to ACE.

*Acetaminophen Time Course*

In previous experiments, embryonic zebrafish exposed to 0.5 μg $L^{-1}$ of ACE were observed at 6, 24, 48 and 72 hpf; most mortality was observed by 24 hpf (Galus et al., 2013). Cyclooxygenase pathways are important in normal gastrulation and segmentation (Cha et al., 2005; Cha et al., 2006b; Grosser et al., 2002), developmental periods completed by 24 hpf (Kimmel et al., 1995). We expected that mortality would be highest from 5-10 (gastrulation) or 11-24 (segmentation), or perhaps be elevated throughout. We completed a detailed time course with observations at 3, 6, 9, 12, 15, 20, 24, 36, 48 and 72 hpf for abnormalities (Figure 5.3) and mortality (Figure 5.4) and found that acetaminophen exposure increased abnormality and mortality rate throughout gastrulation and segmentation, suggesting both processes were impacted by ACE (Figure 5.3). Blastodermal swelling often preceded the onset of pericardial and yolk sac edema, which were frequent abnormalities observe within ACE exposed animals (data not shown). Nearly 51% of all mortality was found in animals with prior observed abnormalities. A third of all embryos died between 6 and 24 hpf; a time frame which encompasses gastrulation and segmentation. Thus, the temporal pattern of abnormalities and mortality are similar to the importance of cyclooxygenase in fish development. It is thought that the edema which develops within this timeframe impedes cell movements preventing formation of the body axes within zebrafish, ultimately leading to mortality (Lepage and Bruce, 2010).

*Cyclooxygenase Activity and Gene Expression*

In mammals, acetaminophen elicits its pharmacological effects through cyclooxygenase inhibition (Hinz and Brune, 2012). Specifically, acetaminophen prevents

COX from converting arachidonic acid to prostaglandin H2, thereby reducing the production of the key precursor to bioactive prostaglandins. The embryonic effects of ACE were thought to be mediated through COX enzymes since the abnormalities and mortality were during COX dependent developmental processes. COX genes are expressed during zebrafish embryogenesis and transcriptional blocking of *cox*-1, but not *cox*-2a expression, caused gastrulation arrest (Grosser et al., 2002). Interestingly, COX derived PGE2 is vital for normal segmentation, and inhibition of zebrafish COX enzymes and morpholino knockdown (10 ng/embryo), lead to shortening of inter-somatic vessels which could cause the onset of edema (Cha et al., 2005; Cha et al., 2006b).

Although *cox*-1, 2a and 2b gene expression has been measured in fish embryos, to our knowledge, COX activity and prostaglandin levels have yet to be documented. While we were unable to determine prostaglandin levels in embryos, likely due to interference from yolk components in prostaglandin bioassays (M. Galus, personal communication), we have measured COX activity in control and ACE exposures zebrafish through development (Figure 5.5). COX activity was detectable throughout development in zebrafish (Figure 5.5A) although the activity is likely much higher in early development. Total COX activity was reported as nmol/min/ml reaction from Figure 5.5; similar to other studies (Bivol et al., 2008; Toller et al., 2010). Yet, the somatic cell contribution to the embryo is very different over these time points and those embryos at early time points have significantly smaller cell mass than those at 72 hpf. Thus, our data underrepresents the COX activity in early embryos and early developmental COX activity is likely much higher than reported here. Normalizing for protein would not correct this challenge; yolk contains significant

protein stores in embryos that complicates traditional measures of total protein content. Yet, micro-dissection of the yolk sac is not practical for embryos less than 24 hpf, the time window most important to this study. Specific inhibitors of mammalian *cox*-1 and *cox*-2 were capable of in vitro inhibition of COX activity in our embryo extracts (Figure 5.5B), supporting the hypothesis that multiple COX genes were present and active in development and that COX function is conserved across vertebrates.

In zebrafish, there are 3 COX genes which control prostaglandin production; *cox-1* which is ubiquitously expressed and *cox*-2a and b which are inducible by cytokines and mitogens (Grosser et al., 2002). It has been shown that *cox*-1 and *cox*-2a are expressed before and throughout gastrulation and segmentation in embryonic zebrafish, and that genes are required for normal developmental processes (Cha et al., 2006a; Grosser et al., 2002; Ishikawa et al., 2007). In these studies, it was found that expression of *cox*-1 was highest, followed closely by *cox*-2a then *cox*-2b. We observed temporal changes in *cox*-1, *cox*-2a and *cox*-2b expression as transcript levels increased during developmental periods of gastrulation and segmentation (Figure 5.6). Overall, the gene expression profiles we report are consistent with previously published literature which identify that *cox*-1, *cox*-2a and *cox*-2b genes are expressed throughout zebrafish development (Cha et al., 2006a; Grosser et al., 2002; Ishikawa et al., 2007).

*Does ACE cause embryo toxicity via inhibition of COX activity?*

In many species, it is well established that prostaglandins play important roles in physiological processes across many stages of life (Brun et al., 2006; Cha et al., 2006b; Gresele et al., 1987; Grosser et al., 2002). These compounds play primary roles during

development as they are involved in oocyte maturation, germ line development and formation of body segments (Gresele et al., 1987; Grosser et al., 2002; Lepage and Bruce, 2010; Lister and Van Der Kraak, 2008). We have demonstrated that embryonic zebrafish exposed to ACE showed elevated rates of abnormality and mortality. Total COX activity was measureable throughout gastrulation and segmentation and this activity was inhibited by both *cox-1* and *cox-2* specific inhibitors. Lastly, expression of *cox*-1, *cox*-2a and *cox*-2b was increased during gastrulation (*cox*-2a and *cox*-2b), and segmentation (*cox*-1 and *cox*-2a). Finally, we co-exposed zebrafish embryos with ACE and PGE2 in order to abolish the pharmacological action of ACE. Supplementation of PGE2 at concentrations of 0.1 – 1 μM was successful at significantly reducing abnormality and mortality rates to levels observed within controls (Figure 5.7, 5.8). Here we provide evidence that exogenous PG supplementation is able to reverse the toxicity of ACE in zebrafish.

*Conclusion*

The results of this study demonstrate that embryonic zebrafish exposed to 0.5- 50 μg $L^{-1}$ of ACE show elevated rates of abnormality and mortality and that these effects are primarily during gastrulation and segmentation. Gene expression data verifies that *cox*-1, *cox*-2a and *cox*-2b were elevated during gastrulation and segmentation and total COX activity measures indicate that the transcripts make functional proteins. Finally, by supplementing exogenous PGE2 we were able to abolish the toxicity observed with ACE exposure alone. Collectively this data provides strong evidence that ACE disrupts the COX-PG pathway at low doses in zebrafish and the mechanism by which NSAIDs elicit developmental toxicity in fish is mechanistically similar to therapeutic effects in mammals.

The fact that ACE is capable of impacting this axis at environmentally relevant concentrations is concerning given that PGs are key bioactive molecules required for homeostatic control at all life stages.

## ACKNOWLEDGEMENTS

Funding was provided by the Natural Sciences and Engineering Research Council of Canada in the form of a Discovery Project and Accelerator Grant to J.Y.W and the Ontario Ministry of Technology and Innovation in the form of an Early Researcher Award to J.Y.W. The Canadian Foundation for Innovation and Ontario Ministry of Research and Innovation provided infrastructure support to J.Y.W. The Swedish Research Council Formas and Carl Tryggers Stiftelse provided support to M.E.J.

**Table 5.1: Embryonic COX activity in zebrafish during development**. Embryos from unexposed parents were collected and COX activity was determined using N=100 embryos per pool, with 3 replicate pools at each time point. Significant differences across time points were determined using a one-way ANOVA with multiple comparisons between time points. Values with different letters are statistically different from one another, $p \leq 0.05$. Data is represetend as mean ± STDEV.

| Hours Post Fertilization (HPF) | COX Activity (nmol/min/mL) |
|---|---|
| 3 | 62 ± 11$^{A}$ |
| 6 | 61.4 ± 10.5$^{A}$ |
| 9 | 56.3 ± 11.8$^{AB}$ |
| 12 | 51.7 ± 12.1$^{AB}$ |
| 15 | 57.3 ± 4.9$^{AB}$ |
| 20 | 54.4 ± 1.5$^{AB}$ |
| 24 | 47.3 ± 5.4 $^{AB}$ |
| 48 | 34.4 ± 6.3$^{B}$ |
| 72 | 35.2 ± 3.4$^{B}$ |

**Figure 5.1**: **Dose response relationship for abnormalities in zebrafish after embryonic exposure to acetaminophen.** Embryos from unexposed parents were immediately exposed to CTL (0) or 0.05, 0.1, 0.5, 1, 5, 10, 20, and 50 µg $L^{-1}$ of aqueous ACE. N=8 for CTL and N=5 plates for all concentrations of ACE. Different letters denote significant differences from CTL; $p \leq 0.05$. Error bars represent SEM.

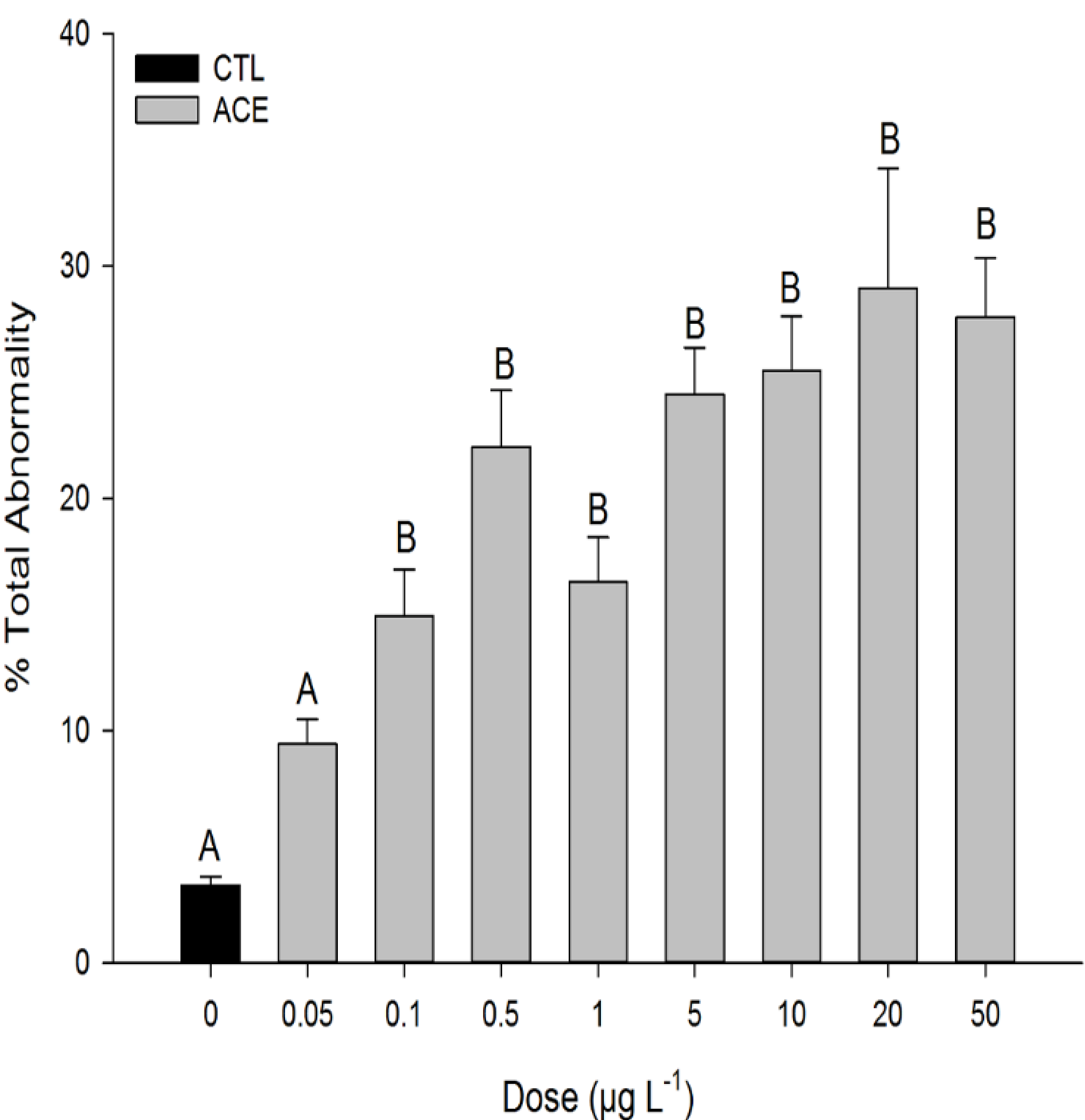

CTL
ACE
% Total Abnormality
0
10
20
30
40
A
A
B
B
B
B
B
B
B
0
0.05
0.1
0.5
1
5
10
20
50
Dose (µg L-1)

**Figure 5.2: Dose response relationship for mortality in zebrafish after embryonic exposure to acetaminophen.** Embryos from unexposed parents were immediately exposed to CTL (0) and 0.05, 0.1, 0.5, 1, 5, 10, 20, and 50 μg $L^{-1}$ of aqueous ACE. N=8 for CTL and N=5 plates for all concentrations of ACE. Different letters denote significant differences from CTL; $p \leq 0.05$. Error bars represent SEM.

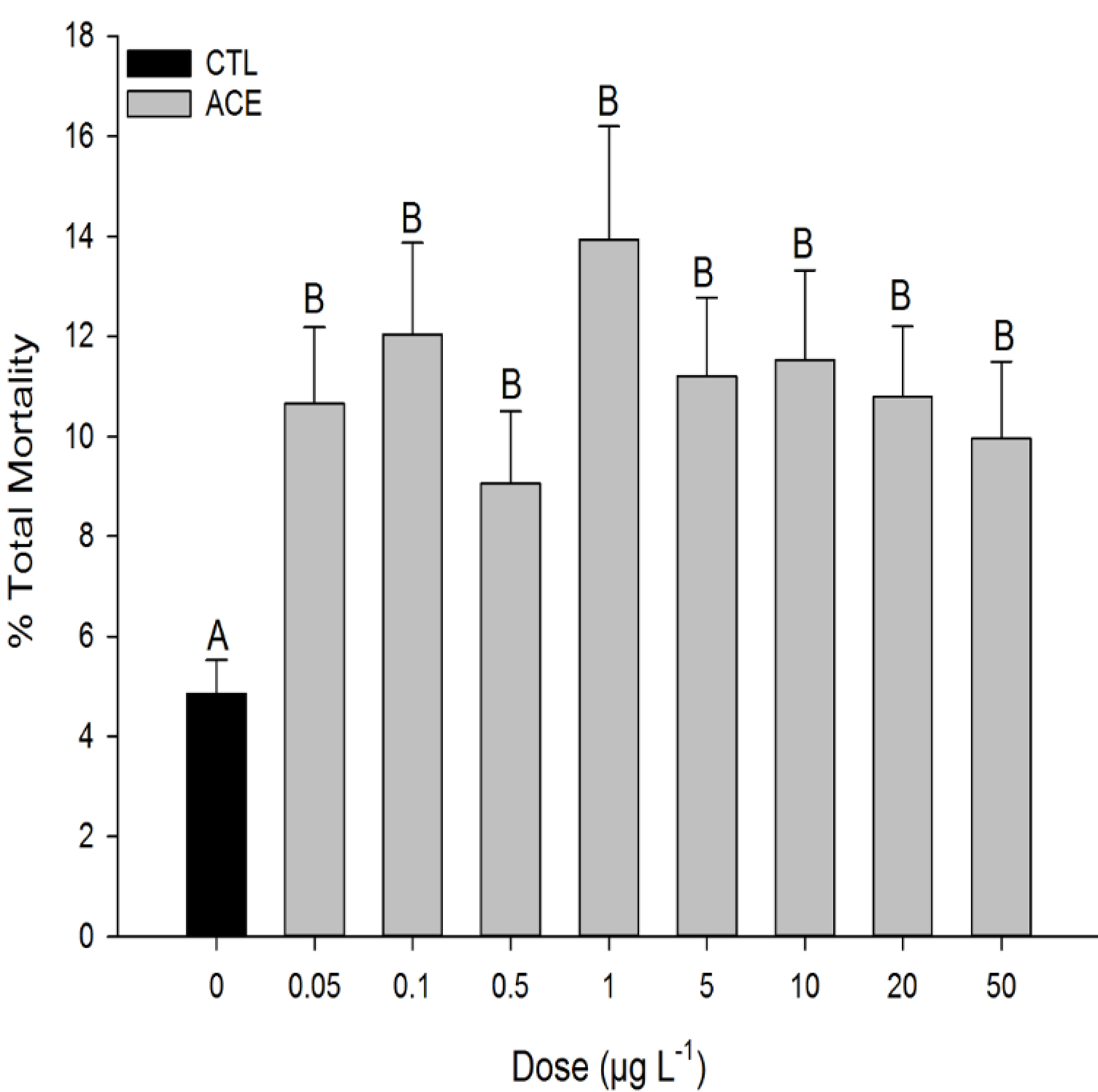

CTL
ACE
% Total Mortality
18
16
14
12
10
8
6
4
2
0
A
B
B
B
B
B
B
B
B
0
0.05
0.1
0.5
1
5
10
20
50
Dose (µg L-1)

**Figure 5.3: Time course for abnormalities during zebrafish development with embryonic exposure to acetaminophen.** Embryos from unexposed parents were immediately exposed to CTL (0) and 0.5 μg $L^{-1}$ of aqueous ACE. N=7 plates for CTL and ACE treatment groups. Error bars represent SEM. CTL is control and ACE is acetaminophen.

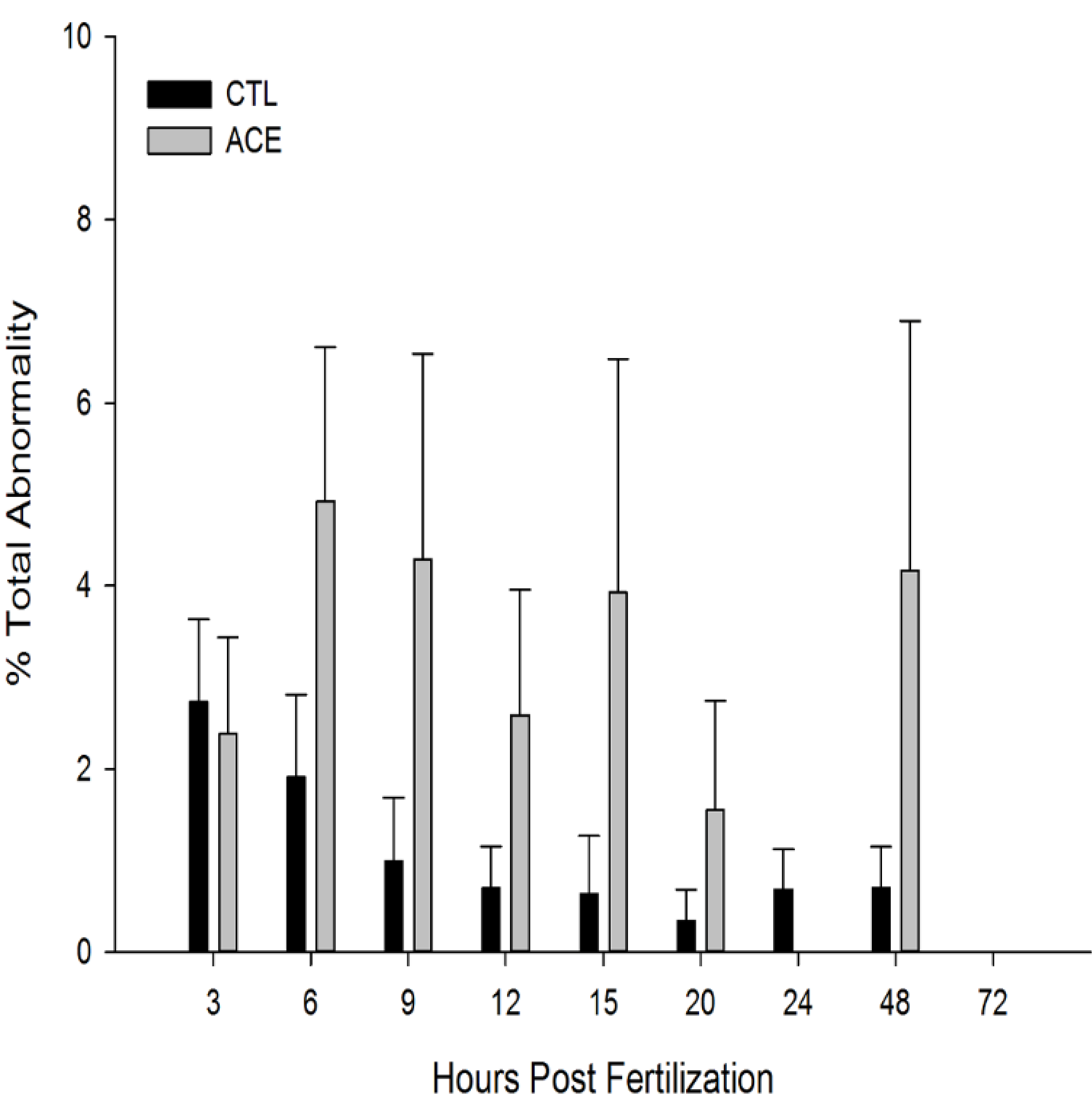

10
8
6
4
2
0
CTL
ACE
% Total Abnormality
3
6
9
12
15
20
24
48
72
Hours Post Fertilization

**Figure 5.4: Time course for mortality during zebrafish development with embryonic exposure to acetaminophen.** Embryos from unexposed parents were immediately exposed to CTL (0) and 0.5 µg $L^{-1}$ of aqueous ACE. N=7 plates for CTL and ACE treatment groups. Error bars represent SEM. CTL is control and ACE is acetaminophen.

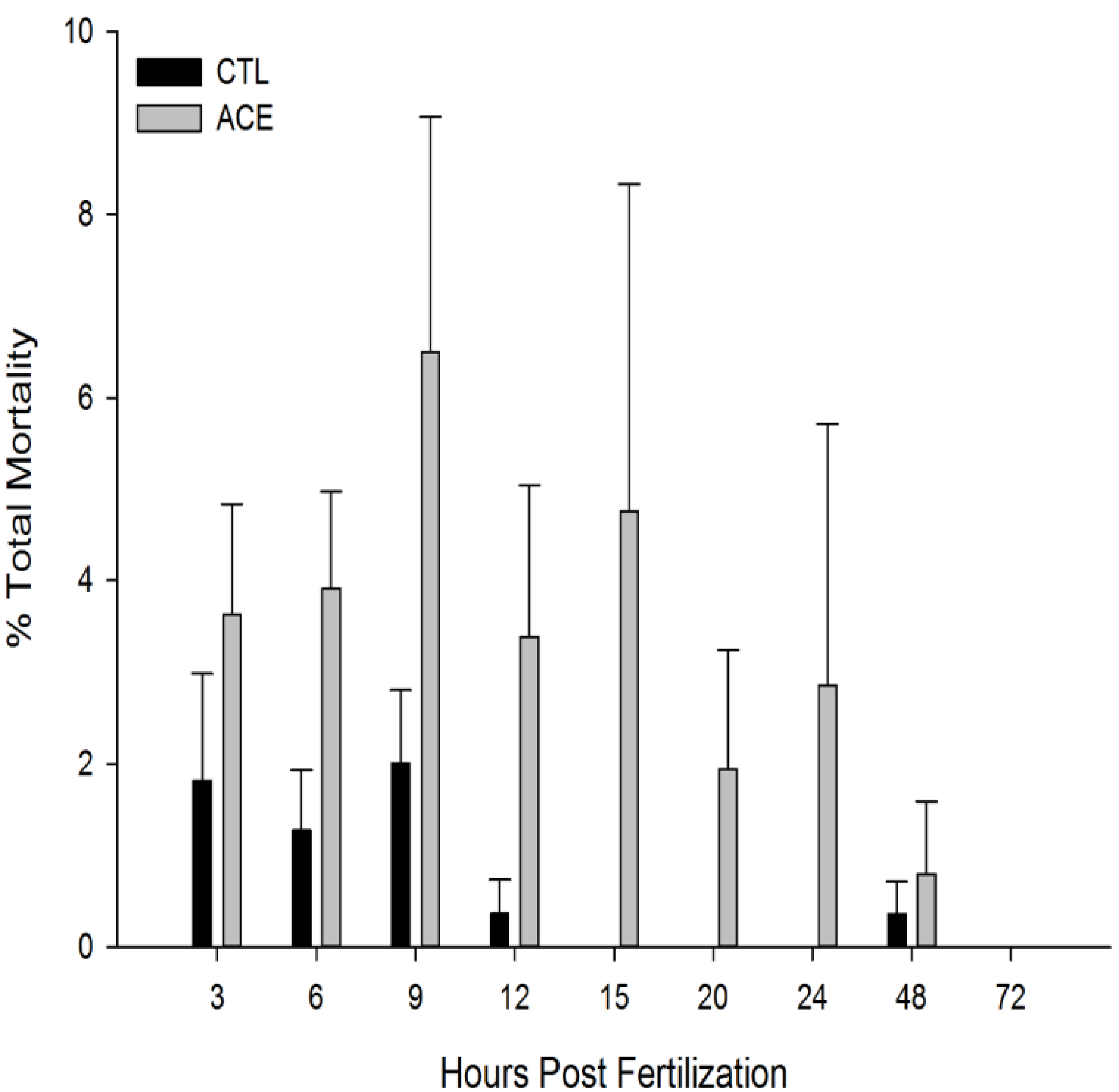

CTL
ACE
% Total Mortality
0
2
4
6
8
10
3
6
9
12
15
20
24
48
72
Hours Post Fertilization

**Figure 5.5: Total cyclooxygenase activity in developing zebrafish with embryonic exposure to acetaminophen.** Embryos from unexposed parents were immediately exposed to CTL (0) and 0.5 μg $L^{-1}$ of aqueous ACE. COX activity was determined in extracts from a pool of 100 embryos, with 3 replicate pools at each time point and dose. Significant differences were determined using t-tests with multiple comparisons; $p \leq 0.05$. A) * denotes significant differences between CTL and ACE samples, within a given timepoint. B) COX activity with mammalian *cox*-1 and *cox*-2 specific inhibitors. CTL COX activity values were set to 100% for each time point. * denotes significant difference of the inhibitor treated sample in relation to its untreated sample, within a given time point. Error bars represent SEM. CTL is control, ACE is acetaminophen, SC is SC-560 (mammalian *cox*-1 specific inhibitor) and DuP is DuP-697 (mammalian *cox*-2 specific inhibitor).

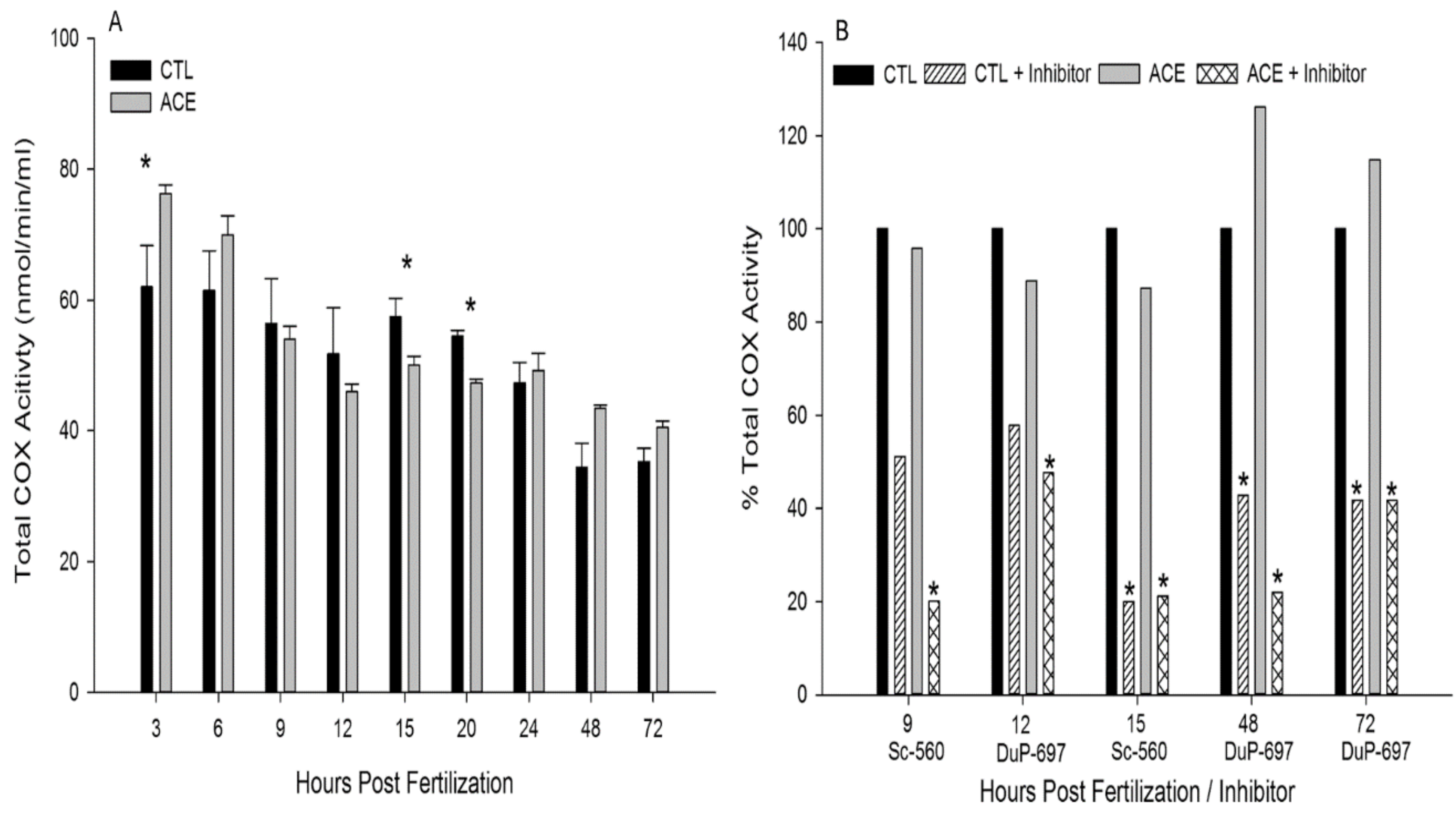

A
CTL
ACE
Total COX Acitivty (nmol/min/ml)
100
80
60
40
20
0
*
*
*
3
6
9
12
15
20
24
48
72
Hours Post Fertilization
B
CTL
CTL + Inhibitor
ACE
ACE + Inhibitor
% Total COX Activity
140
120
100
80
60
40
20
0
9
Sc-560
12
DuP-697
15
Sc-560
48
DuP-697
72
DuP-697
Hours Post Fertilization / Inhibitor

**Figure 5.6: mRNA expression of *cox-1*, *2a* and *2b* in embryonic zebrafish after exposure to 10 nM 6-Formylindolo(3,2-b)carbazole (FICZ).** Embryos from unexposed parents were exposed to 10 nM FICZ or 100 ppm (0.01%) DMSO starting at 2.5 hpf and mRNA levels of *cox-1* (A), *cox-2a* (B) and *cox-2b* (C) were measured at 3 hpf and subsequently every 2 hours until 15 hpf and then at 26 hpf. Samples prior to 2.5 hpf were untreated. Each replicate sample represent 10 pooled embryos, and two replicates (n=2) were analysed at each time point. The shaded area indicates the time before zygotic gene activation, i.e., the time before onset of transcription in the zebrafish embryo (Tadros and Lipshitz, 2009).

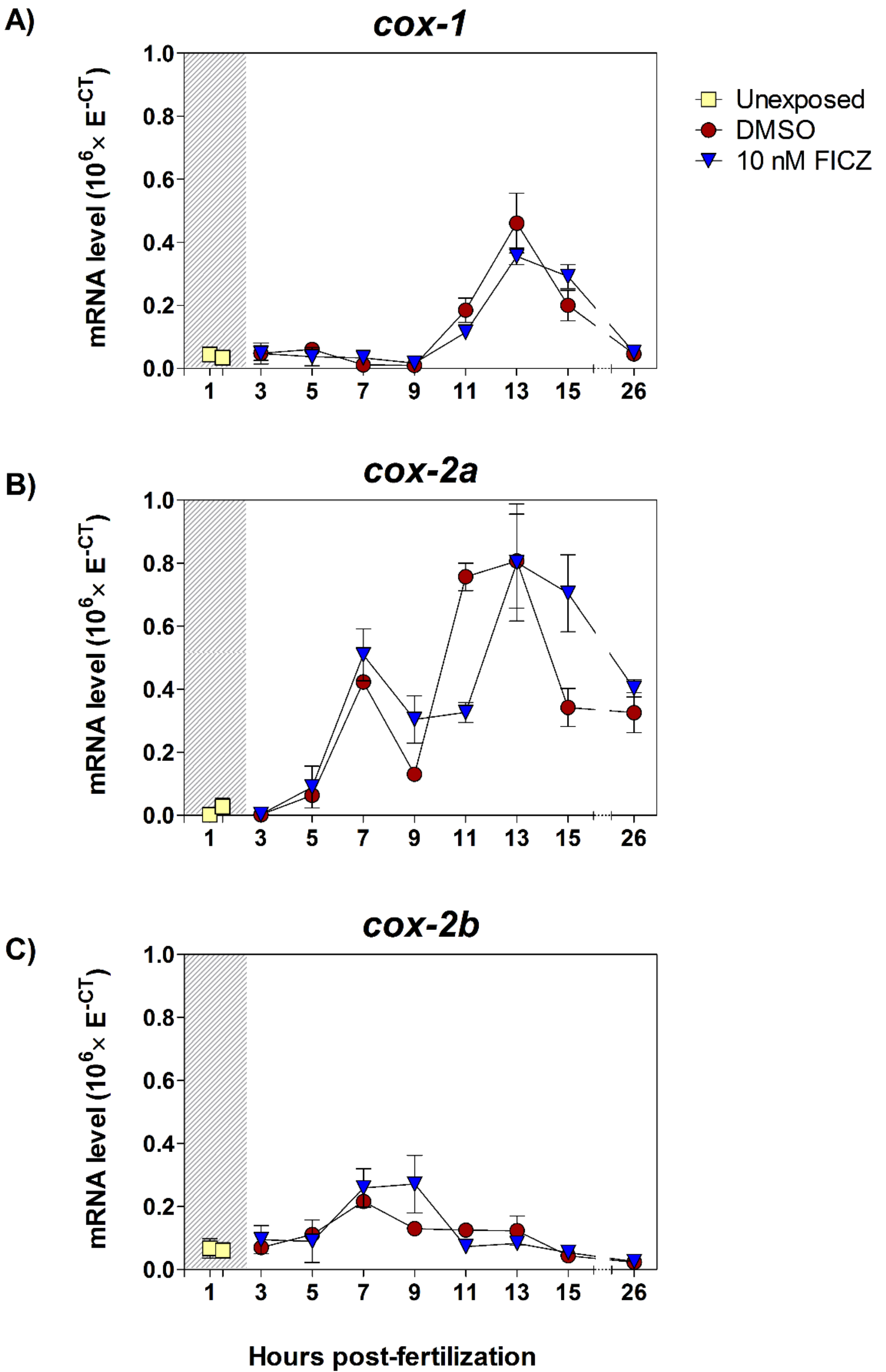

A)
cox-1
B)
cox-2a
C)
cox-2b
mRNA level (10^6× E^-CT)
1.0
0.8
0.6
0.4
0.2
0.0
1
3
5
7
9
11
13
15
26
Hours post-fertilization
Unexposed
DMSO
10 nM FICZ

**Figure 5.7: Total abnormalities in developing zebrafish with embryonic exposure to acetaminophen alone and co-exposed to prostaglandin E2.** Embryos from unexposed parents were immediately exposed to CTL (0), 0.5 μg $L^{-1}$ of aqueous ACE or 0.5 μg $L^{-1}$ of ACE and PGE2 at 0.01, 0.1, 0.5 or 1μM PGE2. N=3 for CTL, ACE alone and each dose of ACE+PGE2. Significant differences were determined using a chi-square test with comparison to CTL. Bars with different letters are statistically different from one another; $p \leq 0.05$. Error bars represent SEM. CTL is contol, ACE is acetaminophen and PGE2 is prostglandin E2.

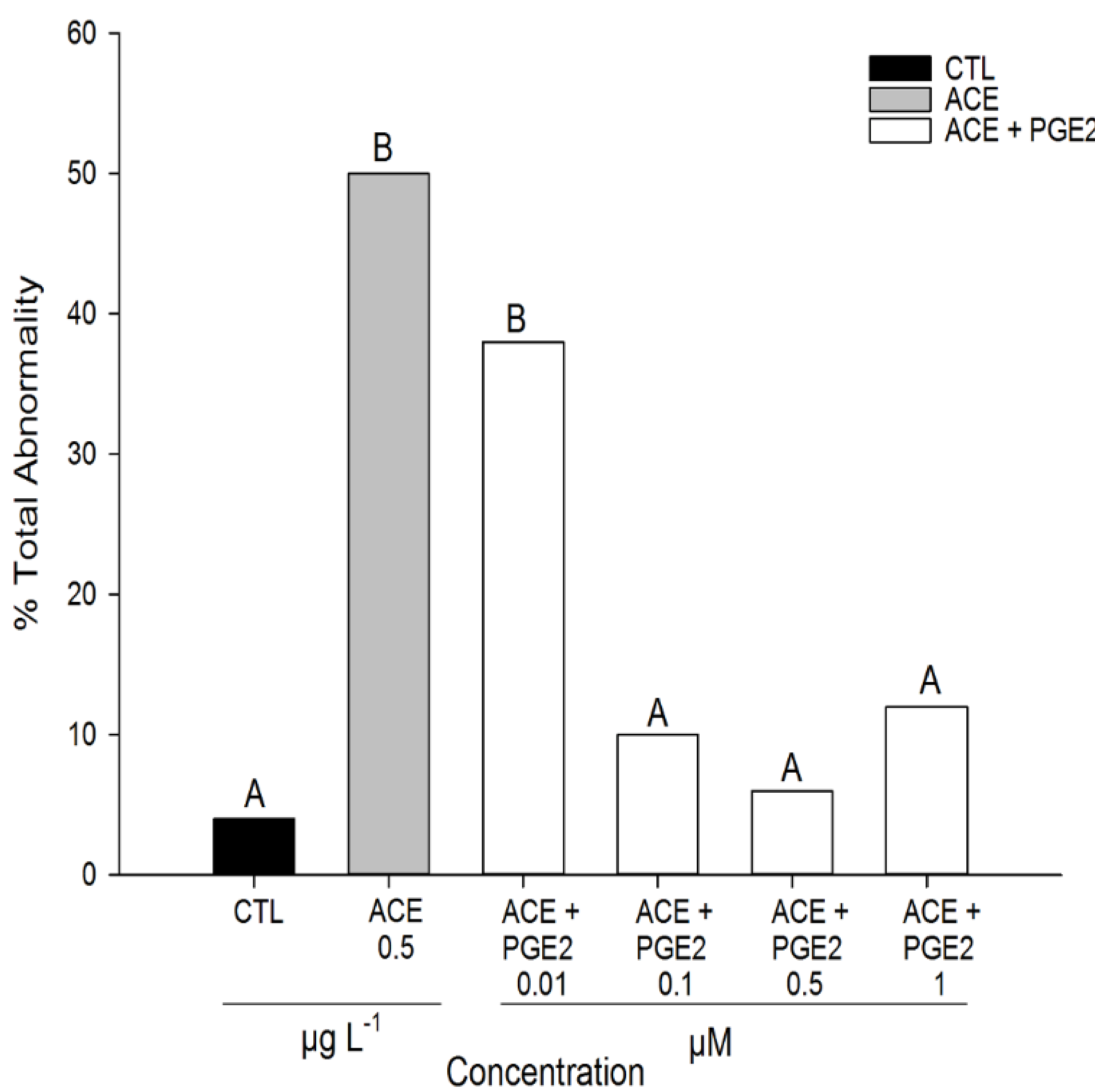
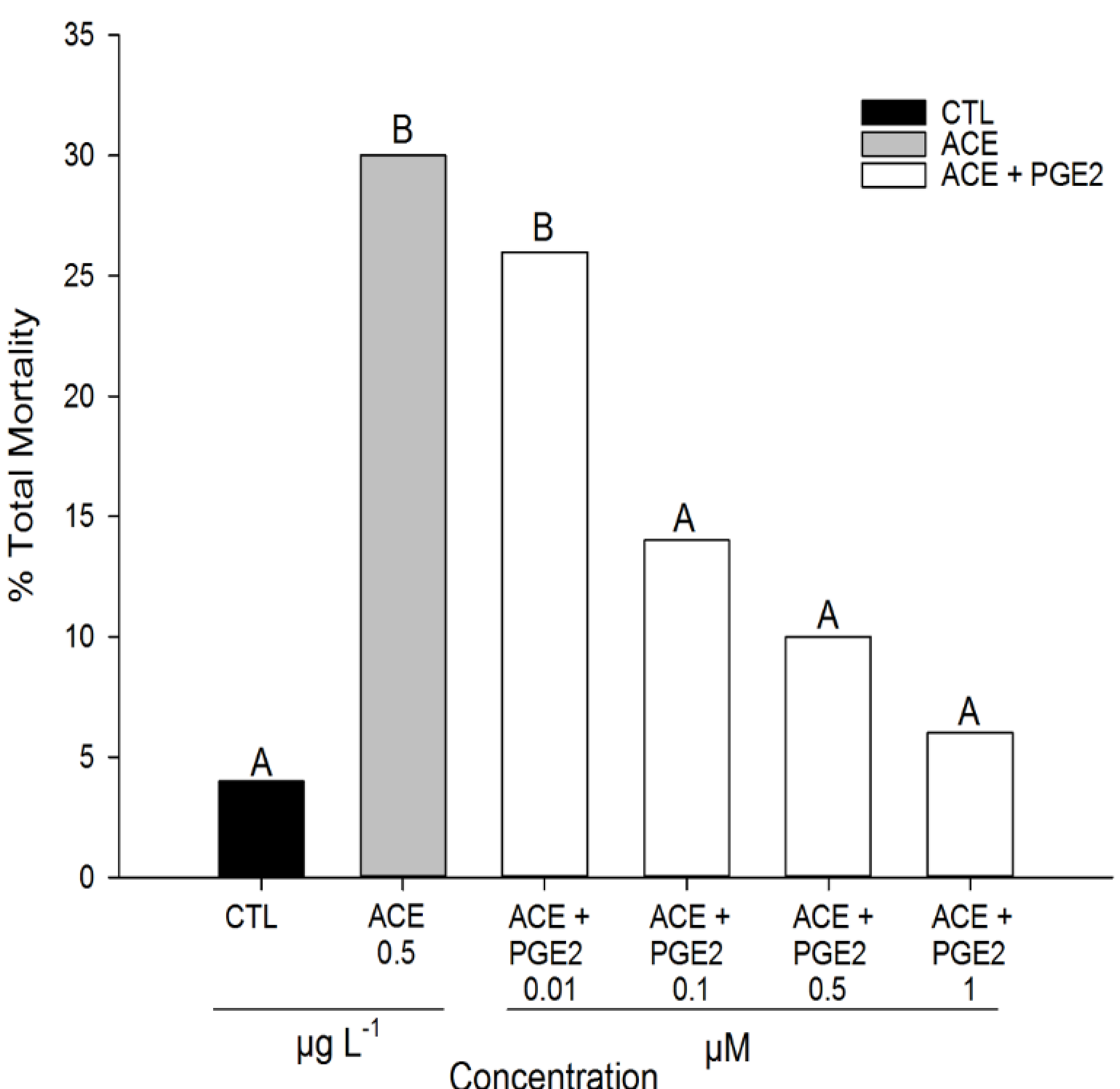

CTL
ACE
ACE + PGE2
% Total Abnormality
60
50
40
30
20
10
0
B
B
A
A
A
A
CTL
ACE 0.5
ACE + PGE2 0.01
ACE + PGE2 0.1
ACE + PGE2 0.5
ACE + PGE2 1
µg L-1
µM
Concentration

**Figure 5.8: Mortality in zebrafish with embryonic exposure to acetaminophen alone and co-exposed with prostaglandin E2.** Embryos from unexposed parents were immediately exposed to CTL (0), 0.5 μg $L^{-1}$ of aqueous ACE or 0.5 μg $L^{-1}$ of ACE and PGE2 at 0.01, 0.1, 0.5 or 1μM PGE2. N=48 embryos for CTL, ACE alone and each dose of ACE+PGE2. Significant differences were determined using a chi-square test with comparison to CTL. Bars with different letter are statistically different from one another; $p \leq 0.05$. Error bars represent SEM. CTL is control, ACE is acetaminophen and PGE2 is prostaglandin E2.


35
30
25
20
15
10
5
0
% Total Mortality
CTL
ACE
ACE + PGE2
B
B
A
A
A
A
CTL
ACE
0.5
ACE +
PGE2
0.01
ACE +
PGE2
0.1
ACE +
PGE2
0.5
ACE +
PGE2
1
µg L-1
µM
Concentration

## REFERENCES

Andreozzi, R., Raffaele, M., Nicklas, P., 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere 50, 1319-1330.

Bivol, L.M., Hultström, M., Gudbrandsen, O.A., Berge, R.K., Iversen, B.M., 2008. Tetradecylthioacetic acid downregulates cyclooxygenase 2 in the renal cortex of two-kidney, one-clip hypertensive rats. Amer. J. of Phys-Reg, Integ & Compart Phys 295, R1866-R1873.

Brun, G.L., Bernier, M., Losier, R., Doe, K., Jackman, P., Lee, H.B., 2006. Pharmaceutically active compounds in Atlantic Canadian sewage treatment plant effluents and receiving waters, and potential for environmental effects as measured by acute and chronic aquatic toxicity. Environ Toxicol Chem 25, 2163-2176.

Buck, A., Sampson, W., Lote, C., Blacklock, N., 1981. The influence of renal prostaglandins on glomerular filtration rate (GFR) and calcium excretion in urolithiasis. British journal of urology 53, 485-491.

Cha, Y.I., Kim, S.-H., Sepich, D., Buchanan, F.G., Solnica-Krezel, L., DuBois, R.N., 2006a. Cyclooxygenase-1-derived PGE2 promotes cell motility via the G-protein-coupled EP4 receptor during vertebrate gastrulation. Genes & dev 20, 77-86.

Cha, Y.I., Kim, S.-H., Solnica-Krezel, L., DuBois, R.N., 2005. Cyclooxygenase-1 signaling is required for vascular tube formation during development. Dev Biol 282, 274-283.

Cha, Y.I., Solnica-Krezel, L., DuBois, R.N., 2006b. Fishing for prostanoids: deciphering the developmental functions of cyclooxygenase-derived prostaglandins. Dev Biol 289, 263-272.

David, A., Pancharatna, K., 2009. Effects of acetaminophen (paracetamol) in the embryonic development of zebrafish, Danio rerio. J. of App Toxicol 29, 597-602.

Elvin, J.A., Yan, C., Matzuk, M.M., 2000. Growth differentiation factor-9 stimulates progesterone synthesis in granulosa cells via a prostaglandin E2/EP2 receptor pathway. PNAS 97, 10288-10293.

Eppig, J.J., 2001. Oocyte control of ovarian follicular development and function in mammals. Reproduction 122, 829-838.

Galus, M., Kirischian, N., Higgins, S., Purdy, J., Chow, J., Rangaranjan, S., Li, H., Metcalfe, C., Wilson, J.Y., 2013. Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. Aquat Toxicol 132, 200-211.

Gao, K., Brandt, I., Goldstone, J.V., Jönsson, M.E., 2011. Cytochrome P450 1A, 1B, and 1C mRNA induction patterns in three-spined stickleback exposed to a transient and a persistent inducer. Compar Bioche & Physiol Part C: Toxicol & Pharma 154, 42-55.

Gierse, J.K., McDonald, J.J., Hauser, S.D., Rangwala, S.H., Koboldt, C.M., Seibert, K., 1996. A single amino acid difference between cyclooxygenase-1 (COX-1) and− 2 (COX-2) reverses the selectivity of COX-2 specific inhibitors. J. of Biol Chem 271, 15810-15814.

Gilchrist, R.B., Ritter, L.J., Armstrong, D.T., 2004. Oocyte–somatic cell interactions during follicle development in mammals. Animal Repro Sci 82, 431-446.

Gresele, P., Arnout, J., Deckmyn, H., Huybrechts, E., Pieters, G., Vermylen, J., 1987. Role of proaggregatory and antiaggregatory prostaglandins in hemostasis. Studies with combined thromboxane synthase inhibition and thromboxane receptor antagonism. J. of Clincal Invest 80, 1435.

Grosser, T., Yusuff, S., Cheskis, E., Pack, M.A., FitzGerald, G.A., 2002. Developmental expression of functional cyclooxygenases in zebrafish. PNAS 99, 8418-8423.

Hinz, B., Brune, K., 2012. Paracetamol and cyclooxygenase inhibition: is there a cause for concern? Annals of Rheumatic Diseases 71, 20-25.

Ishikawa, T.-o., Griffin, K.J., Banerjee, U., Herschman, H.R., 2007. The zebrafish genome contains two inducible, functional cyclooxygenase-2 genes. Biochem & Biophys research commun 352, 181-187.

Jönsson, M.E., Kubota, A., Timme-Laragy, A.R., Woodin, B., Stegeman, J.J., 2012. Ahr2-dependence of PCB126 effects on the swim bladder in relation to expression of CYP1 and cox-2 genes in developing zebrafish. Toxicol & App Pharmacol 265, 166-174.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev Dynamics 203, 253-310.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. Environ Sci & Tech 36, 1202-1211.

Lepage, S.E., Bruce, A., 2010. Zebrafish epiboly: mechanics and mechanisms. Int. J. Dev. Biol 54, 1213-1228.

Lister, A., Van Der Kraak, G., 2008. An investigation into the role of prostaglandins in zebrafish oocyte maturation and ovulation. Gen & Compart Endocrin 159, 46-57.

Martey, C., Baglole, C., Gasiewicz, T., Sime, P., Phipps, R., 2005. The aryl hydrocarbon receptor is a regulator of cigarette smoke induction of the cyclooxygenase and prostaglandin pathways in human lung fibroblasts. Amer. J. of Phys-Lung Cell & Mol Physiol 289, L391-L399.

Parng, C., Anderson, N., Ton, C., McGrath, P., 2004. Zebrafish apoptosis assays for drug discovery. Methods Cell Biol 76, 75-85.

Prescott, S.M., Yost, H.J., 2002. The COXes of Danio: From mechanistic model to experimental therapeutics. PNAS 99, 9084-9086.

Roberts, S.B., Langenau, D.M., Goetz, F.W., 2000. Cloning and characterization of prostaglandin endoperoxide synthase-1 and-2 from the brook trout ovary. Mole & cell Endocrin 160, 89-97.

Rosen, G.D., Birkenmeier, T.M., Raz, A., Holtzman, M.J., 1989. Identification of a cyclooxygenase-related gene and its potential role in prostaglandin formation. Biochem & Biophys research commun 164, 1358-1365.

Rowley, A.F., Vogan, C.L., Taylor, G.W., Clare, A.S., 2005. Prostaglandins in non-insectan invertebrates: recent insights and unsolved problems. J. of Exp Biol 208, 3-14.

Ruijter, J., Ramakers, C., Hoogaars, W., Karlen, Y., Bakker, O., Van den Hoff, M., Moorman, A., 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic acids research 37, e45-e45.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. Nature protocols 3, 1101-1108.

Simmons, D.L., Botting, R.M., Hla, T., 2004. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol reviews 56, 387-437.

Tadros, W., Lipshitz, H.D., 2009. The maternal-to-zygotic transition: a play in two acts. Development 136, 3033-3042.

Toller, I.M., Hitzler, I., Sayi, A., Mueller, A., 2010. Prostaglandin E2 Prevents Helicobacter-Induced Gastric Preneoplasia and Facilitates Persistent Infection in a Mouse Model. Gastroenterology 138, 1455-1467. e1454.

Vane, J., Bakhle, Y., Botting, R., 1998. Cycloxygenase 1 and 2. Annals Rev of Pharmacol & Toxicol 38, 97-120.

Weigt, S., Huebler, N., Braunbeck, T., von Landenberg, F., Broschard, T.H., 2010. Zebrafish teratogenicity test with metabolic activation: Effects of phase I activation of acetaminophen on zebrafish (Danio rerio) embryos. Toxicology 275, 36-49.

Yasojima, K., Schwab, C., McGeer, E.G., McGeer, P.L., 1999. Distribution of cyclooxygenase-1 and cyclooxygenase-2 mRNAs and proteins in human brain and peripheral organs. Brain research 830, 226-236.

Yokoyama, C., Takai, T., Tanabe, T., 1988. Primary structure of sheep prostaglandin endoperoxide synthase deduced from cDNA sequence. FEBS letters 231, 347-351.

Zou, J., Neumann, N., Holland, J., Belosevic, M., Cunnigham, C., Secombes, C., Rowley, A., 1999. Fish macrophages express a cyclo-oxygenase-2 homologue after activation. Biochem. J 340, 153-159

# CHAPTER 6

## GENERAL SUMMARY AND CONCLUSIONS

Over the past few years, the body of literature discussing the toxicity of pharmaceuticals in the aquatic environment has grown. With advancements in technology, we now have the means to detect these compounds at small concentrations in various aqueous media. However, the data discussing the biological response to exposure of these compounds are limited. To date, most of the research has focused on assessing acute toxicity and often the concentrations tested are not indicative of what is detected in the aqueous environment (Corcoran et al., 2010; H. Jones et al., 2005; Heberer, 2002). As such, little was known about the chronic effects of low dose pharmaceutical exposure to fish species. Therefore, the main objectives of this thesis were to assess the toxicity of chronic exposure to environmentally relevant concentrations of single pharmaceuticals and pharmaceutical mixtures including wastewater effluent, on the physiology of zebrafish. The research had a focus on the impacts on reproduction and development. The compounds and concentrations chosen for these studies were based on those frequently detected in receiving waters. These novel findings provide insight into the toxicity associated with chronic, low dose pharmaceutical exposure. Importantly, this data can be used to assess potential effects to native fish species in environmental systems.

### *Compound and Concentration Justification*

With the sheer number of pharmaceuticals and pharmaceutically active compounds present in the aquatic environment, it was quite difficult to decide which compounds warranted investigation. The choice to pursue studies with acetaminophen, carbamazepine,

gemfibrozil and venlafaxine was based on several factors. Firstly, each of these compounds represents a major pharmaceutical class (nonsteroidal anti-inflammatories, anti-epileptics, lipid regulators, anti-depressants) that has been frequently detected in various aqueous media. All four PPCPs were detected in Europe and North America with reported concentrations ranging from high levels of $\mu g\ L^{-1}$ within wastewater effluent (Metcalfe et al., 2010; Metcalfe et al., 2003), dropping to low $\mu g\ L^{-1}$ concentrations within receiving waters. In some cases, concentrations in receiving waters have been reported at low $ng\ L^{-1}$ (Domagalski et al., 2007; Kolpin et al., 2002). The data documenting pharmaceutical contamination provided evidence that these compounds were present at low concentrations, however their impacts on fish at these concentrations were largely unknown.

Acetaminophen was detected at concentrations ranging from 0.0018 – 10 $\mu g\ L^{-1}$ in the environment, with an average concentration of 0.11 $\mu g\ L^{-1}$ (Kolpin et al., 2002; Li et al., 2010). Acetaminophen is a frequently used pharmaceutical and in a recent environmental survey, it was detected in nearly 30% of diverse surface water samples (Kolpin et al., 2002). Carbamazepine is a pharmaceutical that is not heavily used but is rather persistent compared to other PPCPs detected in the environment. It has an average half-life of 82 days and is generally detected between 0.3 – 1.2 $\mu g\ L^{-1}$ (Hoque et al., 2014; Lam et al., 2004; Li et al., 2010). Concentrations of gemfibrozil range from 0.005 – 0.8 $\mu g\ L^{-1}$ (Kolpin et al., 2002; Li et al., 2010) whereas venlafaxine was often detected between 0.05 – 0.9 $\mu g\ L^{-1}$ (Metcalfe et al., 2010).

Current research has shown that exposure to these compounds, or others of the same class, can cause reproductive (Flippin et al., 2007; Ji et al., 2013; Lister et al., 2009;

Runnalls et al., 2013; Skolness et al., 2012; Weinberger II and Klaper, 2014), developmental (David and Pancharatna, 2009; Foran et al., 2004; Hallare et al., 2004; Nassef et al., 2010a; Raldúa et al., 2008), behavioral (Airhart et al., 2007; Nassef et al., 2010b; Painter et al., 2009) and histopathological impacts (Peng et al., 2010; Schwaiger et al., 2004; Triebskorn et al., 2007). However, many of these studies used concentrations that were well above 10 μg $L^{-1}$, the maximum concentration likely found in North American aquatic systems. Although some have shown impacts with environmentally relevant concentrations, chronic low dose studies were largely lacking. With prior data showing these compounds eliciting impacts at higher concentrations, it was hypothesized that they would elicit some effects at low concentrations with a chronic exposure regime. This is of great interest as studying concentrations that are more environmentally relevant allows us to understand the risk of current pharmaceutical contamination in our aquatic ecosystems. Therefore, the choice to conduct exposures at 0.5 and 10 μg $L^{-1}$ was based largely on the range reported in the literature. As pharmaceutical concentrations vary depending on the usage profile and size of the population, these concentrations were not meant to be an exact match to what is found at one wastewater treatment plant, or field site, but to represent concentrations that are reasonable and environmentally relevant.

*Acute versus Chronic Toxicity*

The choice to conduct the experiments in this thesis under a chronic exposure regime was largely based on the endpoints of interest and the concentrations used. I wanted to understand how environmentally relevant concentrations of pharmaceuticals would impact the reproduction and development of fish, therefore I needed to mimic

environmental conditions of exposure. In reality, most fish species are chronically exposed to low doses of pharmaceuticals for a large duration of their lives and it is not often that they are only exposed to a large dose for a short period of time. However, there are examples of fish that are more acutely exposed to low concentrations of chemicals as they transiently move within their full ranges. For example, many native species in South Eastern Lake Ontario use the Cootes Paradise wetland as a spawning and nursery ground and adults are more acutely exposed (days to weeks) to contamination from this site during the breeding season (Mayer et al., 2008; Mayer et al., 2007). For species with such a life history pattern, chronic studies would be predictive of a worst case exposure scenario.

Some PPCPs in the environment can potentially elicit acute toxicity, however as the concentrations are so low, the chances of immediate effects are unlikely. Often times, chronic exposure can lead to an accumulation of sublethal impacts such as delayed time of hatching, changes in heart rate or variations in body size, all of which can have large effects on the physiology of an organism; effects not quantifiable with acute studies (Fraysse et al., 2006; Nagel, 2001). Perhaps the most clear example of the potency of chronic exposure comes from a study conducted by Kidd et al. (2007) who demonstrated that exposure of fathead minnows to the synthetic estrogen 17α-ethynylestradiol for three years, resulted in intersex in males, altered oogenesis in females and a near population crash of the species within the experimental lake; all at a concentration of only 5 - 6 ng $L^{-1}$. Furthermore, chronic exposure can result in the bioaccumulation of pharmaceuticals which can lead to toxic effects long after the source of contamination is removed (Cleuvers, 2004; Mimeault et al., 2005).

From the results in Chapter 2, the exposure of zebrafish to carbamazepine and gemfibrozil did not show impacts on fecundity (Figure 2.2 B, C) until approximately 2-3 weeks after exposure. These impacts may have not been observed if the experiments were carried out under a more acute exposure time. Although chronic toxicity studies are long and difficult to conduct, the data generated from this kind of approach better predicts the toxicity fish experience in natural environments. Chronic exposures can account for the accumulation of subtle changes that can lead to large impacts. Most importantly, they provide for a clearer understanding of the toxicity that fish may experience under real world scenarios.

*Sex Specific Effects on Reproduction*

Reproductive effects were determined in either whole tank group or pairwise matings. In whole tank matings, embryo traps were placed at the bottom of the tank and collected after a spawning event. Although these traps did not collect every embryo produced, as the dimensions of the trap did not cover the entire tank bottom, they are an extremely efficient means to collect embryos from groups of fish in a method that does not require handling and induce significant stress to the animals. Pairwise mating required the netting and transfer of one male and one female to a breeding tank. Fish are separated overnight and then allowed contact just before first light. The handling and isolation of animals involved in pairwise mating is generally thought to induce more stress on the fish. Furthermore, males are often more aggressive towards females that are unresponsive to their courtship display, which could result in damage to the female or even mortality in some cases.

As female zebrafish are asynchronous, batch breeders, it is not expected that a single female would produce a clutch on a daily basis. On average, females will lay eggs once every two days (Eaton and Farley, 1974; Spence and Smith, 2005). Furthermore, when females do mate, they do not release all of their eggs in a single spawning event. Typically, they release 5 – 20 eggs at a time and generally copulate with several males in one spawning session (Skinner and Watt, 2007). Therefore, the benefit of measuring fecundity in a whole tank versus pairwise mating scenario is that tank matings can increase the chances of female spawning. If a male is unsuccessful in stimulating a female, she may choose another mate and potentially lay a clutch. Although pairwise matings provide an excellent means in determining sex specific or recessive traits (Nasiadka and Clark, 2012), they may not be as beneficial as whole tank matings for assessing long term impacts on fecundity. Furthermore, it would not be feasible to conduct a large chronic exposure using a pairwise framework as the amount of time to set up pairs and the stress induced to the animals by handing would be significant.

In Chapters 2 and 3, I assessed the impacts of pharmaceutical exposure on zebrafish fecundity (Figure 2.2, 3.1) in a whole tank mating scenario. This approach minimized handling stress and manipulation of animals during the chronic exposures, and maximized egg production. From the reproductive (Figure 2.2, 3.1) data I inferred that the impacts were female specific as fecundity was reduced; while fertilization rates remained the same. This was supported by altered ovarian histology with exposure to gemfbrozil, carbamazepine, the pharmaceutical mixture and wastewater effluent (Figure 2.5, 2.6, 3.4, 3.5). The altered ovarian histology was an increase in atresia coupled with increased

apoptosis within the theca and granulosa cell layers (Figure 2.6, 3.5). As no histopathology was observed within the testis of males, the histological impacts on the ovary suggested a female specific reduction in fecundity. Yet, exposure to acetaminophen and venlafaxine did not induce histopathological changes to the ovary or testes and impacts on reproduction were still observed. Therefore, the cause for lowered fecundity was not always clear with direct exposure of adult zebrafish.

In Chapter 4, I examined the fecundity of offspring reared from parents, chronically exposed to carbamazepine and gemfibrozil, in pairwise breedings (Figure 4.2). This approach allowed for direct examination of males and females and helped uncover any sex specific effects that may have been unobservable in whole tank matings. Sex specific effects could be determined because exposed animals were paired with an unexposed (control) animal, a reciprocal cross, and compared to fecundity from pairs of unexposed animals and pairs of exposed animals (within treatment crosses). Females from both exposed parents and unexposed parents had similar fecundity when paired with a control male and lower fecundity when paired with the exposed males only, strongly suggested that male exposure history was the controlling factor in measures of fecundity. Altered breeding behaviour was observed with the exposed males and suggested that the reduced fecundity amidst pairs was due to the inability of males to stimulate females to produce a clutch. As male breeding behaviour was not quantified in the adult exposure, I cannot definitively say if male behaviour contributed to the reductions in embryo production. However, the results from Chapter 4 suggest that male behaviour could be a contributing factor in the reduced fecundity observed within adult exposures. It is possible that the

reduced fecundity observed in Chapter 2 and 3 were a combined result of male and female effects. An assessment of breeding behaviour would allow for a more comprehensive understanding of the sex specific effects on reproduction. Examining male breeding behaviour in a pairwise fashion, after chronic exposure, can provide a feasible means for this assessment.

*Common Endpoints of Pharmaceutical Toxicity*

It is quite interesting that all tested pharmaceuticals impacted the rate of developmental mortality, fecundity and histology of the kidney in zebrafish, despite all four having very distinct mechanisms of action. There are considerable amounts of literature documenting similar impacts with similar compounds in various fish species (as reviewed in, Caliman and Gavrilescu, 2009; Corcoran et al., 2010; León-Olea et al., 2014; Trudeau et al., 2011; Trudeau et al., 2005), however to the best of my knowledge, this is the first study to show that chronic, low concentration PPCP exposure, with the exception of estrogens, has impacts on multiple organ systems in fish.

In Chapters 2 and 3, I used the incidence of mortality and abnormality throughout embryogenesis to quantify the toxicity of direct pharmaceutical exposure to zebrafish embryos. The results obtained from these experiments suggested an inverted U-shaped dose response (Figure 2.3, 3.2). However, in Chapter 5, a more detailed examination of the toxicity of acetaminophen on embryonic development was conducted and the inclusion of more doses revealed that the dose response relationship for acetaminophen was closer to a linear or rise to maximum response rather than the previously thought U-shaped curve (Figure 5.1, 5.2.) Assessing developmental toxicity using mortality and abnormalities as

endpoints may not be the best choice moving forward as these are rather severe effects. The increases observed in mortality were only 2 - 4% larger in exposed fish (excluding acetaminophen) over controls and elevated abnormality was only observed with acetaminophen. It may be more fruitful to look at subtle changes throughout development such as alterations in body size which may lead to consequences in food acquisition, predator avoidance and mate selection (Osse et al., 1997). Mortality may be a very suitable endpoint for examining pharmaceutical mixtures because the highest mortality was with the pharmaceutical mixture and yet wastewater effluent did not induce very high mortality levels.

In the laboratory, pharmaceutical impacts on reproduction are often considered in association with other physiological impacts as reductions in fecundity can stem from alterations in hormone concentrations (Arcand-Hoy and Benson, 1998; Blüm and Fiedler, 1965; Jobling et al., 1996), changes in behaviour (Jones and Reynolds, 1997; Scott and Sloman, 2004), or impacts on gene expression (Mennigen et al., 2009; Mennigen et al., 2010). Tight control of internal hormone concentrations, particularly of estrogen and testosterone, are required for normal reproductive processes in fish (Kime, 1999; Schreck et al., 2001). In Chapter 2, I demonstrated that carbamazepine exposure at 0.5 $\mu g\ L^{-1}$ significantly reduced circulating concentrations of 11-ketotestosterone in male and female zebrafish (Figure 2.7). Although other compounds also largely reduced these concentrations, the large variation coupled with small sample size, decreased statistical power needed to robustly test for differences amongst treatment groups. During sampling, I was uncertain as to how much plasma I would need to optimize these measurements, thus

I made sampling pools consisting of 25 fish. Reevaluating these methods, it is apparent that I could have pooled less fish and increased my sample size to increase replication and statistical power. Although I cannot definitively say if the reduced hormone concentrations contributed to the reductions in fecundity, it is possible as other studies that have documented changes in hormone concentrations observed impacts on reproduction (Ji et al., 2013; Skolness et al., 2012; Winter et al., 2008). In Chapter 4, I demonstrated that offspring raised from parents chronically exposed to carbamazepine and gemfibrozil had altered breeding behaviour (Figure 4.3, 4.4) which resulted in impacts on reproduction (Figure 4.1, 4.2). Appropriate courtship displays are used between fish to identify that they are ready to reproduce (Darrow and Harris, 2004) and deviations from these behaviours often result in reduced breeding success (Bell, 2001). Collectively, it is apparent that while fecundity is a measure of reproduction, there are many other physiological processes and pathways that are involved in regulating reproduction. To better understand the complexity of pharmaceutical exposure on reproduction, it would be fruitful to examine those processes that are involved in its regulation. Lastly, the reproductive effects documented in Chapters 2 - 4 suggest that fish exposed to PPCPs in the wild could be susceptible to population level impacts (Bhatia et al., 2014; Flippin et al., 2007; Martinović et al., 2008; Mennigen et al., 2010; Mennigen et al., 2008; Nash et al., 2004; Olsén et al., 2014; Schreck et al., 2001; Sebire et al., 2008; Winter et al., 2008) and that laboratory studies provide an efficient means quantify the severity of effects that could occur in natural settings.

The effects observed with the kidney could lead to impacts on fish health. In fish, much like in other vertebrates, the liver is the primary organ responsible for metabolism of

exogenous compounds whereas the kidney is involved in excretion (Brusle and Anadon, 1996; Lackner, 1998; Miller, 1987; Pritchard and Miller, 1980). Unique to fish, the renal system has an extensive portal circulation system (Pritchard and Miller, 1980). This ensures that the kidney tubules are exposed to a higher fraction of cardiac circulation allowing for an adequate reabsorption of key ions and solutes (Pritchard and Miller, 1980). Freshwater teleosts such as zebrafish, are hyperosmotic to their surrounding environment meaning that they are passively loosing ions through the gill (Evans, 2008). In order to compensate for this, fish are constantly producing large volumes of dilute urine to maintain ionic balance (Evans, 2008). However, under xenobiotic load the increased kidney activity and portal circulation can be detrimental as it would increase the exposure time of kidney tubules to exogenous compounds, potentially increasing the level of toxicity. Exposure to all pharmaceutical compounds severely impacted the morphology of the kidney. Although it is a nearly impossible task to measure kidney function, in zebrafish, it is likely that from the severity of the damage observed, normal kidney function was impacted. Chronic pharmaceutical exposure could have altered the integrity of the kidney tubules potentially making them leakier or reducing the efficiency of filtering the compounds out of the body, resulting in an increased internal concentration; exacerbating the toxicity of the compound(s). It would be fruitful to assess the impacts these drugs have on the kidney in larger model species such as rainbow trout.

*Mixture Toxicity*

Most toxicological studies focus on the effects from exposure to a single compound, often at high concentrations (Yang, 1994). However, in the natural environment, fish are

generally exposed to low doses of a wide range of contaminants (Benotti and Brownawell, 2007; Benotti et al., 2008; Brown et al., 2006; Kolpin et al., 2002; Metcalfe et al., 2010; Metcalfe et al., 2003). In order to better understand the toxicity fish experience in the wild, it is important to begin assessing the implications of pharmaceutical mixtures on fish health. Not only can these studies describe the physiological impacts but they can also help clarify drug-drug interactions or the issues of species sensitivity (De Zwart and Posthuma, 2005).

Although there are several studies documenting mixture toxicity with invertebrate model species (Borgmann et al., 2007; DeLorenzo and Fleming, 2008; Dietrich et al., 2010) and cell cultures (Alsop and Wood, 2013; Fernández et al., 2013), there are very few studies that have looked at the impacts of mixture toxicity in fish. Parrott and Bennie (2009) exposed fathead minnows to six pharmaceuticals (naproxen, gemfibrozil, diclofenac, ibuprofen, salicylic acid, acetaminophen) and one personal care product (triclosan). The concentrations tested were within environmental levels (0.01 – 1 μg $L^{-1}$) however there were no impacts observed on the development or fecundity of fish (Parrott and Bennie, 2009). The only observable difference was in the frequency of abnormalities detected in offspring, which was nearly double what was observed within control animals (Parrott and Bennie, 2009). From the pharmaceuticals chosen, five were NSAIDs with gemfibrozil being the only fibrate. It appeared that the authors may have expected additive toxicity given that the NSAIDs would have the same mechanism of action, however as single compound exposures were not conducted, determining potential mixture interactions were not possible.

The results obtained in Chapter 3 help contribute to the small number of studies that have addressed the toxicological impacts of mixtures to fish species. The data generated in this chapter showed that pharmaceuticals of different classes impacted common endpoints and suggested interaction effects because there were more severe effects with the pharmaceutical mixture than expected based on single compound exposures. However, due to the limited number of doses tested it is difficult to accurately determine potential interactions and distinguish between additivity or synergism within the mixture. These data would benefit from the completion of binary mixtures with each compound and then tertiary mixtures. This approach would allow for a clearer understanding of the compound(s) primarily responsible for altering the toxicity of the mixture. Furthermore, as pharmaceutical concentrations vary depending on usage and the surrounding population, it is unreasonable to assume that all compounds would be present in the environment at equal concentrations. Therefore, varying relative concentrations of compounds within the mixture need to be considered.

## CONCLUSIONS

The main objectives of this thesis were to assess the toxicity of chronic exposure to environmentally relevant concentrations of single pharmaceuticals, pharmaceutical mixtures and wastewater effluent on the physiology of zebrafish; with a focus on developmental and reproductive endpoints. At the time this thesis began, there was little published data describing the consequences of environmentally relevant concentrations of pharmaceuticals on the health of fish. Through the approaches discussed in Chapters 2 – 5, I have expanded this knowledge base by:

1. Demonstrating that environmentally relevant concentrations of acetaminophen, carbamazepine, gemfibrozil and venlafaxine reduced the fecundity of adult zebrafish, impacted the morphology of the kidney and increased mortality throughout embryogenesis.

2. Demonstrating that effects observed with single compound exposures are found with a mixture of the same four pharmaceuticals and diluted wastewater effluent.

3. Demonstrating that chronic, parental exposure to carbamazepine and gemfibrozil impacted the fecundity, breeding behaviour and sperm morphology and velocity in male offspring.

4. Demonstrating that the mechanism of developmental toxicity for acetaminophen in fish was similar to the therapeutic mode of action in mammals.

Overall, this thesis advances the knowledge of the effects of environmentally relevant concentrations of pharmaceuticals in fish. This data is novel and provides suggested endpoints for use for future studies and can be used to extrapolate the types of impacts native fish species may experience in natural settings.

**FUTURE DIRECTIONS**

Future studies should be aimed at examining the toxicological impacts of mixtures to fish species. The data in this thesis suggests that mixture exposures can potentially be more harmful than single compound exposures. It is important to note that exposures in the environment are never to one compound and data generated from mixture studies can provide a clearer understanding of the toxicity fish may experience in the real world. The caveat of mixture research, moving forward, is how to test the toxicity of mixtures given the sheer number of pharmaceuticals and pharmaceutically active compounds. Of course it

is unreasonable to test every drug in every combination, but emphasis should be placed on those pharmaceuticals that are frequently detected in environmental surveys as well as those which are resistant to degradation. Future studies would also benefit from a systematic approach moving from single compound exposures to more complex mixtures (binary, tertiary, etc.) as well as varying the concentrations of the compounds within mixtures. This methodology would allow for clear identification of the compound(s) that is responsible for altering the toxicity of the others in the mixture. Lastly, focus should also be placed on understanding the drug-drug interactions within fish species exposed to mixtures. These interactions may be extremely complex and involve multiple physiological processes and enzymes that are well understood in mammals but not yet well characterized in fish. By understanding the mechanisms of drug-drug interactions, it would allow for a clearer identification of the most potent compounds and ultimately provide a more comprehensive understanding of the toxicity to fish. These findings could then be extrapolated to natural settings in order to better estimate the implications of environmentally relevant concentrations of pharmaceuticals on the health of native fish species.

## REFERENCES

Airhart, M.J., Lee, D.H., Wilson, T.D., Miller, B.E., Miller, M.N., Skalko, R.G., 2007. Movement disorders and neurochemical changes in zebrafish larvae after bath exposure to fluoxetine (PROZAC). Nerotoxicol Teratol 29, 652-664.

Alsop, D., Wood, C.M., 2013. Metal and pharmaceutical mixtures: Is ion loss the mechanism underlying acute toxicity and widespread additive toxicity in zebrafish? Aquat Toxicol 140, 257-267.

Arcand-Hoy, L.D., Benson, W.H., 1998. Fish reproduction: an ecologically relevant indicator of endocrine disruption. Environ Toxicol Chem 17, 49-57.

Bell, A.M., 2001. Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined stickleback, *Gasterosteus aculeatus*. An Behav 62, 775-780.

Benotti, M.J., Brownawell, B.J., 2007. Distributions of pharmaceuticals in an urban estuary during both dry- and wet-weather conditions. Environ Sci Technol 41, 5795-5802.

Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder, S.A., 2008. Pharmaceuticals and endocrine disrupting compounds in US drinking water. Environ Sci Technol 43, 597-603.

Bhatia, H., Kumar, A., Ogino, Y., Du, J., Gregg, A., Chapman, J., McLaughlin, M.J., Iguchi, T., 2014. Effects of the commercial antiandrogen flutamide on the biomarkers of reproduction in male Murray rainbowfish (*Melanotaenia fluviatilis*). Environ Toxicol Chem 33, 1098-1107.

Blüm, V., Fiedler, K., 1965. Hormonal control of reproductive behavior in some cichlid fish. Gen Comp Endocr 5, 186-196.

Borgmann, U., Bennie, D., Ball, A., Palabrica, V., 2007. Effect of a mixture of seven pharmaceuticals on *Hyalella azteca* over multiple generations. Chemosphere 66, 1278-1283.

Brown, K.D., Kulis, J., Thomson, B., Chapman, T.H., Mawhinney, D.B., 2006. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. Sci Total Environ 366, 772-783.

Brusle, J., Anadon, G., 1996. The structure and function of fish liver. Fish Morph 77-93.

Caliman, F.A., Gavrilescu, M., 2009. Pharmaceuticals, personal care products and endocrine disrupting agents in the environment–a review. Clean - Soil, Air, Water 37, 277-303.

Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotox Environ Safe 59, 309-315.

Corcoran, J., Winter, M.J., Tyler, C.R., 2010. Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. Crit Rev Toxicol 40, 287-304.
Darrow, K.O., Harris, W.A., 2004. Characterization and development of courtship in zebrafish, *Danio rerio*. Zebrafish 1, 40-45.

David, A., Pancharatna, K., 2009. Effects of acetaminophen (paracetamol) in the embryonic development of zebrafish, *Danio rerio*. J Appl Toxicol 29, 597-602.

De Zwart, D., Posthuma, L., 2005. Complex mixture toxicity for single and multiple species: Proposed methodologies. Environ Toxicol Chem 24, 2665-2676.

DeLorenzo, M.E., Fleming, J., 2008. Individual and mixture effects of selected pharmaceuticals and personal care products on the marine phytoplankton species *Dunaliella tertiolecta*. Arch Environ Con Tox 54, 203-210.

Dietrich, S., Ploessl, F., Bracher, F., Laforsch, C., 2010. Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in *Daphnia magna*–A multigenerational study. Chemosphere 79, 60-66.

Domagalski, J., Belitz, K., Furlong, E., 2007. Occurrence of pharmaceutical and other organic compounds in ground water in ten regions of California, USA, AGU Spring Meeting Abstracts, p. 03.

Eaton, R.C., Farley, R.D., 1974. Spawning cycle and egg production of zebrafish, *Brachydanio rerio*, in the laboratory. Copeia, 195-204.

Evans, D.H., 2008. Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. Am J Phsyiol - Reg I 295, R704-R713.

Fernández, C., Carbonell, G., Babín, M., 2013. Effects of individual and a mixture of pharmaceuticals and personal-care products on cytotoxicity, EROD activity and ROS production in a rainbow trout gonadal cell line (RTG-2). J Appl Toxicol 33, 1203-1212.

Flippin, J.L., Huggett, D., Foran, C.M., 2007. Changes in the timing of reproduction following chronic exposure to ibuprofen in Japanese medaka, *Oryzias latipes*. Aquat Toxicol 81, 73-78.

Foran, C.M., Weston, J., Slattery, M., Brooks, B.W., Huggett, D.B., 2004. Reproductive assessment of Japanese medaka (*Oryzias latipes*) following a four-week fluoxetine (SSRI) exposure. Arch Environ Con Tox 46, 511-517.

Fraysse, B., Mons, R., Garric, J., 2006. Development of a zebrafish 4-day embryo-larval bioassay to assess toxicity of chemicals. Ecotox Environ Safe 63, 253-267.

H. Jones, O., Voulvoulis, N., Lester, J., 2005. Human pharmaceuticals in wastewater treatment processes. Crit Rev Environ Sci Technol 35, 401-427.

Hallare, A., Köhler, H.-R., Triebskorn, R., 2004. Developmental toxicity and stress protein responses in zebrafish embryos after exposure to *diclofenac* and its solvent, DMSO. Chemosphere 56, 659-666.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol Lett 131, 5-17.

Hoque, M.E., Cloutier, F., Arcieri, C., McInnes, M., Sultana, T., Murray, C., Vanrolleghem, P.A., Metcalfe, C.D., 2014. Removal of selected pharmaceuticals, personal care products and artificial sweetener in an aerated sewage lagoon. Sci Total Environ.

Ji, K., Liu, X., Lee, S., Kang, S., Kho, Y., Giesy, J.P., Choi, K., 2013. Effects of non-steroidal anti-inflammatory drugs on hormones and genes of the hypothalamic-pituitary-gonad axis, and reproduction of zebrafish. J Hazard Mater 254, 242-251.

Jobling, S., Sumpter, J.P., Sheahan, D., Osborne, J.A., Matthiessen, P., 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environ Toxicol Chem 15, 194-202.

Jones, J.C., Reynolds, J.D., 1997. Effects of pollution on reproductive behaviour of fishes. Rev Fish Biol Fisheries 7, 463-491.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. PNAS 104, 8897-8901.

Kime, D.E., 1999. A strategy for assessing the effects of xenobiotics on fish reproduction. Sci Total Environ 225, 3-11.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. Environ Sci Technol 36, 1202-1211.

Lackner, R., 1998. “Oxidative stress” in fish by environmental pollutants, Fish ecotoxicology. Springer, pp. 203-224.

Lam, M.W., Young, C.J., Brain, R.A., Johnson, D.J., Hanson, M.A., Wilson, C.J., Richards, S.M., Solomon, K.R., Mabury, S.A., 2004. Aquatic persistence of eight pharmaceuticals in a microcosm study. Environ Toxicol Chem 23, 1431-1440.

León-Olea, M., Martyniuk, C.J., Orlando, E.F., Ottinger, M.A., Rosenfeld, C.S., Wolstenholme, J.T., Trudeau, V.L., 2014. Current concepts in neuroendocrine disruption. Gen Comp Endocr.

Li, H., Helm, P.A., Metcalfe, C.D., 2010. Sampling in the Great Lakes for pharmaceuticals, personal care products, and endocrine-disrupting substances using the passive polar organic chemical integrative sampler. Environ Toxicol Chem 29, 751-762.

Lister, A., Regan, C., Van Zwol, J., Van Der Kraak, G., 2009. Inhibition of egg production in zebrafish by fluoxetine and municipal effluents: A mechanistic evaluation. Aquat Toxicol 95, 320-329.

Martinović, D., Blake, L.S., Durhan, E.J., Greene, K.J., Kahl, M.D., Jensen, K.M., Makynen, E.A., Villeneuve, D.L., Ankley, G.T., 2008. Reproductive toxicity of vinclozolin in the fathead minnow: Confirming an anti-androgenic mode of action. Environ Toxicol Chem 27, 478-488.

Mayer, T., Bennie, D., Rosa, F., Palabrica, V., Rekas, G., Schachtschneider, J., Marvin, C., 2008. Dispersal of contaminants from municipal discharges as evidenced from sedimentary records in a Great Lakes coastal wetland, Cootes Paradise, Ontario. J Great Lakes Res 34, 544-558.

Mayer, T., Bennie, D., Rosa, F., Rekas, G., Palabrica, V., Schachtschneider, J., 2007. Occurrence of alkylphenolic substances in a Great Lakes coastal marsh, Cootes Paradise, ON, Canada. Environ Poll 147, 683-690.

Mennigen, J.A., Harris, E.A., Chang, J.P., Moon, T.W., Trudeau, V.L., 2009. Fluoxetine affects weight gain and expression of feeding peptides in the female goldfish brain. Regul Peptides 155, 99-104.

Mennigen, J.A., Lado, W.E., Zamora, J.M., Duarte-Guterman, P., Langlois, V.S., Metcalfe, C.D., Chang, J.P., Moon, T.W., Trudeau, V.L., 2010. Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, *Carassius auratus*. Aquat Toxicol 100, 354-364.

Mennigen, J.A., Martyniuk, C.J., Crump, K., Xiong, H., Zhao, E., Popesku, J., Anisman, H., Cossins, A.R., Xia, X., Trudeau, V.L., 2008. Effects of fluoxetine on the reproductive axis of female goldfish (*Carassius auratus*). Physiol Genomics 35, 273-282.

Metcalfe, C.D., Chu, S., Judt, C., Li, H., Oakes, K.D., Servos, M.R., Andrews, D.M., 2010. Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. Environ Toxicol Chem 29, 79-89.

Metcalfe, C.D., Miao, X.S., Koenig, B.G., Struger, J., 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ Toxicol Chem 22, 2881-2889.

Miller, D.S., 1987. Aquatic models for the study of renal transport function and pollutant toxicity. Environ Health Persp 71, 59.

Mimeault, C., Woodhouse, A., Miao, X.-S., Metcalfe, C., Moon, T., Trudeau, V., 2005. The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, *Carassius auratus*. Aquat Toxicol 73, 44-54.

Nagel, R., 2001. The embryo test with the Zebrafish *Danio rerio*--a general model in ecotoxicology and toxicology. Altex 19, 38-48.

Nash, J.P., Kime, D.E., Van der Ven, L.T., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R., 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Environ Health Persp, 1725-1733.

Nasiadka, A., Clark, M.D., 2012. Zebrafish breeding in the laboratory environment. ILAR Journal 53, 161-168.

Nassef, M., Kim, S.G., Seki, M., Kang, I.J., Hano, T., Shimasaki, Y., Oshima, Y., 2010a. In *ovo* nanoinjection of triclosan, diclofenac and carbamazepine affects embryonic development of medaka fish (*Oryzias latipes*). Chemosphere 79, 966-973.

Nassef, M., Matsumoto, S., Seki, M., Khalil, F., Kang, I.J., Shimasaki, Y., Oshima, Y., Honjo, T., 2010b. Acute effects of triclosan, diclofenac and carbamazepine on feeding performance of Japanese medaka fish (*Oryzias latipes*). Chemosphere 80, 1095-1100.

Olsén, K.H., Ask, K., Olsén, H., Porsch-Hällström, I., Hallgren, S., 2014. Reprint of Effects of the SSRI citalopram on behaviours connected to stress and reproduction in Endler guppy, *Poecilia wingei*. Aquat Toxicol 151, 97-104.

Osse, J., Van Den Boogaart, J., Van Snik, G., Van Der Sluys, L., 1997. Priorities during early growth of fish larvae. Aquaculture 155, 249-258.

Painter, M.M., Buerkley, M.A., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., Furlong, E.T., Schultz, M.M., Schoenfuss, H.L., 2009. Antidepressants at environmentally

relevant concentrations affect predator avoidance behavior of larval fathead minnows (*Pimephales promelas*). Environ Toxicol Chem 28, 2677-2684.

Parrott, J., Bennie, D., 2009. Life-cycle exposure of fathead minnows to a mixture of six common pharmaceuticals and triclosan. J Toxicol Environ Health A 72, 633-641.

Peng, H.-C., Wang, Y.-H., Wen, C.-C., Wang, W.-H., Cheng, C.-C., Chen, Y.-H., 2010. Nephrotoxicity assessments of acetaminophen during zebrafish embryogenesis. Comp Biochem Phys C 151, 480-486.

Pritchard, J., Miller, D., 1980. Teleost kidney in evaluation of xenobiotic toxicity and elimination, Federation proceedings, pp. 3207-3212.

Raldúa, D., André, M., Babin, P.J., 2008. Clofibrate and gemfibrozil induce an embryonic malabsorption syndrome in zebrafish. Toxicol App Pharm 228, 301-314.

Runnalls, T.J., Beresford, N., Losty, E., Scott, A.P., Sumpter, J.P., 2013. Several synthetic progestins with different potencies adversely affect reproduction of fish. Environ Sci Technol 47, 2077-2084.

Schreck, C.B., Contreras-Sanchez, W., Fitzpatrick, M.S., 2001. Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture 197, 3-24.

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., Negele, R., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part I: histopathological alterations and bioaccumulation in rainbow trout. Aquat Toxicol 68, 141-150.

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquat Toxicol 68, 369-392.

Sebire, M., Allen, Y., Bersuder, P., Katsiadaki, I., 2008. The model anti-androgen flutamide suppresses the expression of typical male stickleback reproductive behaviour. Aquat Toxicol 90, 37-47.

Skinner, A.M., Watt, P.J., 2007. Strategic egg allocation in the zebra fish, *Danio rerio*. Behav Ecol 18, 905-909.

Skolness, S.Y., Durhan, E.J., Jensen, K.M., Kahl, M.D., Makynen, E.A., Villeneuve, D.L., Ankley, G.T., 2012. Effects of gemfibrozil on lipid metabolism, steroidogenesis, and reproduction in the fathead minnow (*Pimephales promelas*). Environ Toxicol Chem 31, 2615-2624.

Spence, R., Smith, C., 2005. Male territoriality mediates density and sex ratio effects on oviposition in the zebrafish, *Danio rerio*. Animal Behaviour 69, 1317-1323.

Triebskorn, R., Casper, H., Scheil, V., Schwaiger, J., 2007. Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). Anal Bioanal Chem 387, 1405-1416.

Trudeau, V.L., Kah, O., Bourguignon, J.-P., 2011. Neuroendocrine disruption: the emerging concept. J Toxicol Environ Health B 14, 267-269.
Trudeau, V.L., Metcalfe, C.D., Mimeault, C., Moon, T.W., 2005. Pharmaceuticals in the environment: Drugged fish? Biochem Mol Biol Fish 6, 475-493.

Weinberger II, J., Klaper, R., 2014. Environmental concentrations of the selective serotonin reuptake inhibitor fluoxetine impact specific behaviors involved in reproduction, feeding and predator avoidance in the fish *Pimephales promelas* (fathead minnow). Aquat Toxicol 151, 77-83.

Winter, M.J., Lillicrap, A.D., Caunter, J.E., Schaffner, C., Alder, A.C., Ramil, M., Ternes, T.A., Giltrow, E., Sumpter, J.P., Hutchinson, T.H., 2008. Defining the chronic impacts of atenolol on embryo-larval development and reproduction in the fathead minnow (*Pimephales promelas*). Aquat Toxicol 86, 361-369.

Yang, R., 1994. Introduction to the toxicology of chemical mixtures, YangRSH, ed, Toxicology of Chemical Mixtures. Academic, San Diego, CA USA,, pp. 1-10.

# APPENDIX

The following is a list of supplemental tables and figures used to append the data discussed in this thesis. Supplemental tables S1 – S4 are used in Chapter 2. Supplemental tables S5 – S8 are used in Chapter 3. Supplemental figure S1 is used in Chapter 2

**Supplemental Table S1: Measured aqueous pharmaceutical concentrations.** Pharmaceutical concentrations ($\mu gL^{-1}$, mean ± standard deviation, N=3 tanks) measures in water samples taken at 10 min, 24, 28 and 72 hrs after water changeout. Nominal doses were 0 (Ctrl), 0.5 (Low) and 10 (High) $\mu gL^{-1}$ for all pharmaceuticals.

| **Pharmaceutical** | **Time** | | | | **Exposure Tank** |
|---|---|---|---|---|---|
| | **10 min** | **24 hr** | **48 hr** | **72 hr** | |
| **Acetaminophen** | 0.68 ± 0.07 | 0.81 ± 0.13 | 0.90 ± 0.39 | 0.84 ± 0.39 | Ctrl |
| | 1.06 ± 0.14 | 0.91 ± 0.13 | 0.87 ± 0.43 | 0.61 ± 0.17 | Low |
| | 6.61 ± 0.42 | 1.97 ± 1.97 | 0.88 ± 0.29 | 0.62 ± 0.09 | High |
| **Carbamazepine** | 0.06 ± 0.02 | 0.08 ± 0.01 | 0.03 ± 0.02 | 0.07 ± 0.01 | Ctrl |
| | 0.72 ± 0.04 | 0.67 ± 0.06 | 0.70 ± 0.08 | 0.18 ± 0.05 | Low |
| | 9.20 ± 1.03 | 10.97 ± 0.19 | 10.37 ± 1.00 | 1.60 ± 0.20 | High |
| **Gemfibrozil** | 0.04 ± 0.02 | 0.03 ± 0.02 | 0.09 ± 0.01 | 0.01 ±0.003 | Ctrl |
| | 0.64 ± 0.05 | 0.51 ± 0.10 | 0.58 ± 0.33 | 0.13 ± 0.03 | Low |
| | 10.18 ± 2.22 | 5.74 ± 5.16 | 6.78 ± 4.57 | 1.22 ± 0.19 | High |
| **Venlafaxine** | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | Ctrl |
| | 0.27 ± 0.03 | 0.29 ± 0.01 | 0.29 ± 0.02 | 0.06 ±0.007 | Low |
| | 5.45 ± 0.23 | 6.11 ± 0.53 | 5.21 ± 0.77 | 0.64 ± 0.12 | High |

**Supplemental Table S2: Histopathological damage in the kidney of zebrafish exposed to pharmaceuticals.** Adult fish were exposed for 6 weeks to 0 (Control), 0.5 (Low) and 10 (High) µg L-1 of aqueous acetaminophen (ACE), carbamazepine (CBZ), gemfibrozil (GEM) and venlafaxine (VEN). Whole zebrafish were embedded, serially sectioned at 5µm, and stained with hematoxylin and eosin prior to screening under 40X magnification and standard light microscopy. Control fish from all experiments were not statistically different in the incidence of histopathology or mean score; therefore control data is pooled from all exposures. Histopathological scores are described by Bernet et al. (1999). Intensity of damage was scored on a 5 point scale with 0/1 representing no or very minimal damage to the kidney tubule, 2 – moderate loss of cytoplasm within the kidney tubule and nuclei vacuolization, 3 – very limited cytoplasm within the kidney tubule with noncentral nuclei positioning and 4 – complete loss of native structure and staining integrity; see materials and methods in chapter 2 for details. All exposures showed a statistically significant increase in the incidence of histopathology and mean histopathological score, when compared to controls; $p < 0.05$. Statistical analyses were performed using the control animals within the experimental exposure and not on the pooled data shown below.

| **Sex** | **Treatment** | **N** | **Score** | | | | | **Incidence** |
|---|---|---|---|---|---|---|---|---|
| | | | **0** | **1** | **2** | **3** | **4** | |
| ***Males*** | Control | 87 | 86 | 1 | 0 | 0 | 0 | 0.01 |
| | Ace Low | 20 | 4 | 5 | 8 | 3 | 0 | 0.8 |
| | Ace High | 18 | 3 | 4 | 4 | 7 | 0 | 0.83 |
| | Cbz Low | 16 | 1 | 5 | 4 | 6 | 0 | 0.94 |
| | Cbz High | 19 | 2 | 2 | 9 | 5 | 1 | 0.89 |
| | Gem Low | 22 | 8 | 8 | 4 | 2 | 0 | 0.64 |
| | Gem High | 20 | 2 | 6 | 5 | 6 | 1 | 0.9 |
| | Ven Low | 10 | 2 | 4 | 4 | 0 | 0 | 0.8 |
| | Ven High | 19 | 4 | 9 | 6 | 0 | 0 | 0.79 |
| ***Females*** | Control | 102 | 98 | 3 | 1 | 0 | 0 | 0.04 |
| | Ace Low | 19 | 4 | 5 | 7 | 3 | 0 | 0.79 |
| | Ace High | 33 | 13 | 5 | 14 | 1 | 0 | 0.61 |
| | Cbz Low | 32 | 5 | 11 | 14 | 2 | 0 | 0.84 |
| | Cbz High | 27 | 4 | 4 | 10 | 9 | 0 | 0.85 |
| | Gem Low | 24 | 8 | 6 | 10 | 0 | 0 | 0.67 |
| | Gem High | 20 | 5 | 6 | 4 | 5 | 0 | 0.75 |
| | Ven Low | 35 | 11 | 15 | 8 | 1 | 0 | 0.69 |
| | Ven High | 30 | 12 | 9 | 9 | 0 | 0 | 0.60 |

**Supplemental Table S3: Histopathological damage in the liver of zebrafish exposed to pharmaceuticals.** Adult fish were exposed for 6 weeks to 0 (Control), 0.5 (Low) and 10 (High) µg L-1 of aqueous acetaminophen (ACE), carbamazepine (CBZ), gemfibrozil (GEM) and venlafaxine (VEN). Whole zebrafish were embedded, serially sectioned at 5µm, and stained with hematoxylin and eosin prior to screening under 40X magnification and standard light microscopy. Control fish from all experiments were not statistically different in the incidence of histopathology or mean score; therefore control data is pooled from all exposures. Histopathological scores are adapted from Bernet et al. (Bernet et al. 1999). Intensity of damage was scored on a 3 point scale with 0 representing no or very minimal damage to the liver, 1 – hepatocyte cytoplasmic loss with nuclei vacuolization and 2 – hepatocyte fibrosis or severe glycogen depletion; see materials and methods in chapter 2 for details. Those exposures with a statistically significant increase in mean histopathological score and incidence, over controls, are noted with a † and *, respectively; $p < 0.05$. Statistical analyses were performed using the control animals within the experimental exposure and not on the pooled data shown below.

| **Sex** | **Treatment** | **N** | **Score** | | | **Incidence** |
|---|---|---|---|---|---|---|
| | | | **0** | **1** | **2** | |
| ***Males*** | Control | 87 | 87 | 0 | 0 | 0 |
| | Ace Low† | 20 | 16 | 4 | 0 | 0.2* |
| | Ace High | 18 | 17 | 1 | 0 | 0.06 |
| | Cbz Low | 16 | 16 | 0 | 0 | 0 |
| | Cbz High | 19 | 19 | 0 | 0 | 0 |
| | Gem Low | 22 | 19 | 3 | 0 | 0.14 |
| | Gem High† | 20 | 13 | 4 | 3 | 0.4* |
| | Ven Low | 10 | 10 | 0 | 0 | 0 |
| | Ven High | 19 | 19 | 0 | 0 | 0 |
| ***Females*** | Control | 102 | 102 | 0 | 0 | 0 |
| | Ace Low | 19 | 19 | 0 | 0 | 0 |
| | Ace High† | 33 | 28 | 5 | 0 | 0.15* |
| | Cbz Low | 32 | 32 | 0 | 0 | 0 |
| | Cbz High | 27 | 27 | 0 | 0 | 0 |
| | Gem Low† | 24 | 19 | 5 | 0 | 0.21* |
| | Gem High | 20 | 18 | 2 | 0 | 0.1 |
| | Ven Low | 35 | 35 | 0 | 0 | 0 |
| | Ven High | 30 | 29 | 1 | 0 | 0.03 |

**Supplemental Table S4: Histopathological damage in the gonads of zebrafish exposed to pharmaceuticals**. Adult fish were exposed for 6 weeks to 0 (Control), 0.5 (Low) and 10 (High) µg $L^{-1}$ of aqueous acetaminophen (ACE), carbamazepine (CBZ), gemfibrozil (GEM) and venlafaxine (VEN). Whole zebrafish were embedded, serially sectioned at 5µm, and stained with hematoxylin and eosin prior to screening under 40X magnification and standard light microscopy. Control fish from all experiments were not statistically different in the incidence of histopathology or mean score; therefore control data is pooled from all exposures. Histopathological scores are adapted from Bernet et al. (1999). Intensity of damage was scored on a 3 point scale. Males and females scored 0 display no or very minimal damage to the gonad, 1 – degradation of the sperm body or an increase in stromal tissue within the oocyte and 2 – calcification or fibrosis of the testicular lumen or smaller mature oocytes with vitellogenin degradation; see materials and methods in chapter 2 for details. Those exposures with a statistically significant increase in mean histopathological score and incidence, over controls, are noted with a † and *, respectively; $p < 0.05$. Statistical analyses were performed using the control animals within the experimental exposure and not on the pooled data shown below.

| **Sex** | **Treatment** | **N** | **Score** | | | **Incidence** |
|---|---|---|---|---|---|---|
| | | | **0** | **1** | **2** | |
| ***Males*** | Control | 87 | 84 | 3 | 0 | 0.03 |
| | Ace Low | 20 | 19 | 0 | 1 | 0.05 |
| | Ace High | 18 | 16 | 2 | 0 | 0.11 |
| | Cbz Low | 16 | 15 | 0 | 1 | 0.06 |
| | Cbz High | 19 | 18 | 1 | 0 | 0.05 |
| | Gem Low | 22 | 21 | 1 | 0 | 0.05 |
| | Gem High | 20 | 19 | 1 | 0 | 0.05 |
| | Ven Low | 10 | 10 | 0 | 0 | 0 |
| | Ven High | 19 | 19 | 0 | 0 | 0 |
| ***Females*** | Control | 102 | 95 | 7 | 0 | 0.07 |
| | Ace Low | 19 | 14 | 5 | 0 | 0.26 |
| | Ace High | 33 | 26 | 5 | 2 | 0.21 |
| | Cbz Low† | 32 | 13 | 18 | 1 | 0.59* |
| | Cbz High† | 27 | 8 | 17 | 2 | 0.7* |
| | Gem Low† | 24 | 9 | 15 | 0 | 0.63* |
| | Gem High† | 20 | 4 | 13 | 3 | 0.8* |
| | Ven Low | 35 | 31 | 4 | 0 | 0.11 |
| | Ven High | 30 | 27 | 3 | 0 | 0.1 |

**Supplemental Table S5: Mean concentrations (µg $L^{-1}$; N=3) of selected pharmaceutical compounds in triplicate samples collected from a WWTP.** Samples were collected in October, 2006 and 2008 as 24-hr composite samples of untreated and treated wastewater and as grab samples after the primary and secondary clarifiers. COT = Cotinine, CAF = Caffeine, CBZ = Carbamazepine, TMP = Trimethoprim, ACE = Acetaminophen, NPX = Naproxen, IBP = Ibuprofen, GEM = Gemfibrozil, SFP = Sulfapyridine, SMX = Slufamethoxazole, TCS = Triclosan, TCC = Triclocarban, VEN = Venlafaxine, CLP = Citalopram, FLX = Fluoxetine, STA = Sertraline, ANL = Atenolol, NDL = Nadolol, PPL = Propranolol.

| | | ***2006*** | | | | | | ***2008*** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Influent | 2º Clarifier | Effluent | % Removal | Influent | 1º Clarifier | 2º Clarifier | Effluent | % Removal |
| **Base/Neutrals** | COT | 1.32 | 0.08 | 0.03 | 98 | 1.4 | 1.31 | 1.01 | 0.18 | 87 |
| | CAF | 29.28 | 0.95 | 0.22 | >99 | 47.07 | 27.83 | 37.45 | 0.09 | >99 |
| | CBZ | 0.37 | 0.36 | 0.38 | 0 | 0.28 | 0.24 | 0.26 | 0.28 | 0 |
| | TMP | 0.25 | 0.19 | 0.19 | 21 | 0.28 | 0.24 | 0.22 | 0.17 | 40 |
| **Acidics** | ACE | 56 | 0.03 | 0.05 | >99 | 45.54 | 39.87 | 31.11 | 0.16 | >99 |
| | NPX | 2.48 | 0.29 | 0.02 | 98 | 3.75 | 4.37 | 3.67 | 0.66 | 82 |
| | IBP | 5.71 | 0.03 | <DL | >99 | 6.22 | 6.62 | 4.78 | 0.87 | 86 |
| | GEM | 0.1 | 0.03 | 0.01 | 89 | 0.14 | 0.11 | 0.11 | 0.07 | 46 |
| **Sulfonamides** | SFP | NA | NA | NA | NA | 0.13 | 0.14 | 0.12 | 0.07 | 45 |
| | SMX | NA | NA | NA | NA | 0.57 | 0.58 | 0.44 | 0.16 | 72 |
| **Antibacterials** | TCS | NA | NA | NA | NA | 1.48 | 0.7 | 0.86 | 0.18 | 88 |
| | TCC | NA | NA | NA | NA | 0.3 | 0.11 | 0.18 | 0.06 | 79 |
| **Antidepressants** | VEN | NA | NA | NA | NA | 0.32 | 0.29 | 0.21 | 0.28 | 13 |
| | CLP | NA | NA | NA | NA | 0.16 | 0.1 | 0.78 | 0.12 | 24 |
| | FLX | <LOQ | 0.02 | 0.01 | NC | 0.02 | 0.008 | 0.009 | 0.01 | 20 |
| | STA | NA | NA | NA | NA | 0.03 | 0.012 | 0.014 | 0.009 | 69 |
| **Beta-Blockers** | ANL | NA | NA | NA | NA | 0.84 | 0.78 | 0.32 | 0.52 | 38 |
| | NDL | NA | NA | NA | NA | 0.04 | 0.03 | 0.02 | 0.02 | 50 |
| | PPL | NA | NA | NA | NA | 0.27 | 0.42 | 0.18 | 0.28 | NC |
| **EDCs** | BPA | 43 | 18.3 | 16.1 | 63 | NA | NA | NA | NA | NA |
| | Estrone | 0.05 | 0.06 | 0.04 | 28 | NA | NA | NA | NA | NA |

**Supplemental Table S6: Mean (±SD) measured aqueous concentrations (μg $L^{-1}$; N=3) of acetaminophen (ACE), carbamazepine (CBZ), gemfibrozil (GEM) and venlafaxine (VEN) in samples of tank water collected during exposures of zebrafish to mixtures (MIX) and treated wastewater (WWE).** All concentrations are the mean ± standard deviation for N=3 tanks. Pharmaceutical and effluent concentrations were renewed every three days. Water samples were taken at 10 min, 24, 48 and 72 hrs after the first dose, in a single cycle, for the mixture exposure and at 10 min and 24 hrs post dose for the effluent trial. Nominal doses were 0 (Ctrl), 0.5 (Low) and 10 (High) μg $L^{-1}$ for MIX treatments and 0 (Ctrl), 5% (Low) and 25% (High) for WWE treatments.

| Experiment | Dose | Time (hr) | ACE | CBZ | GEM | VEN |
|---|---|---|---|---|---|---|
| **MIX** | Control | 0.17 | <LOQ | 0.20±0.06 | <LOQ | <LOQ |
| | | 24 | <LOQ | 0.08±0.07 | 0.11±0.18 | 0.06±0.10 |
| | | 48 | <LOQ | 0.04±0.07 | 0.18±0.15 | 0.11±0.19 |
| | | 72 | <LOQ | 0.06±0.11 | 0.37±0.09 | 0.13±0.22 |
| | Low | 0.17 | <LOQ | 0.56±0.05 | <LOQ | 0.04±0.07 |
| | (0.5 µg $L^{-1}$) | 24 | 0.09±0.16 | 0.55±0.20 | 0.31±0.54 | <LOQ |
| | | 48 | 0.05±0.08 | 0.48±0.26 | 0.29±0.32 | 0.04±0.07 |
| | | 72 | 0.03±0.05 | 0.19±0.31 | 0.35±0.17 | <LOQ |
| | High | 0.17 | 1.02±1.40 | 10.8±0.39 | 0.0±0.0 | 2.20±3.63 |
| | (10 µg $L^{-1}$) | 24 | 1.65±2.40 | 11.9±2.11 | 12.4±0.03 | 4.97±4.71 |
| | | 48 | 1.29±1.12 | 10.8±1.18 | 5.86±2.17 | 4.77±5.11 |
| | | 72 | 0.03±0.03 | 1.31±0.48 | 0.74±0.16 | 6.77±10.5 |
| **WWE** | Control | 0.17 | <LOQ | <LOQ | <LOQ | <LOQ |
| | | 24 | <LOQ | <LOQ | <LOQ | <LOQ |
| | Low | 0.17 | <LOQ | 0.09±0.07 | 0.28±0.08 | <LOQ |
| | (5%) | 24 | <LOQ | 0.08±0.07 | 0.28±0.03 | <LOQ |
| | High | 0.17 | 0.04±0.01 | 0.13±0.11 | 0.23±0.09 | 0.11±0.01 |
| | (25%) | 24 | 0.03±0.03 | 0.12±0.10 | 0.22±0.06 | 0.09±0.01 |

<LOQ = Below limits of quantitation.

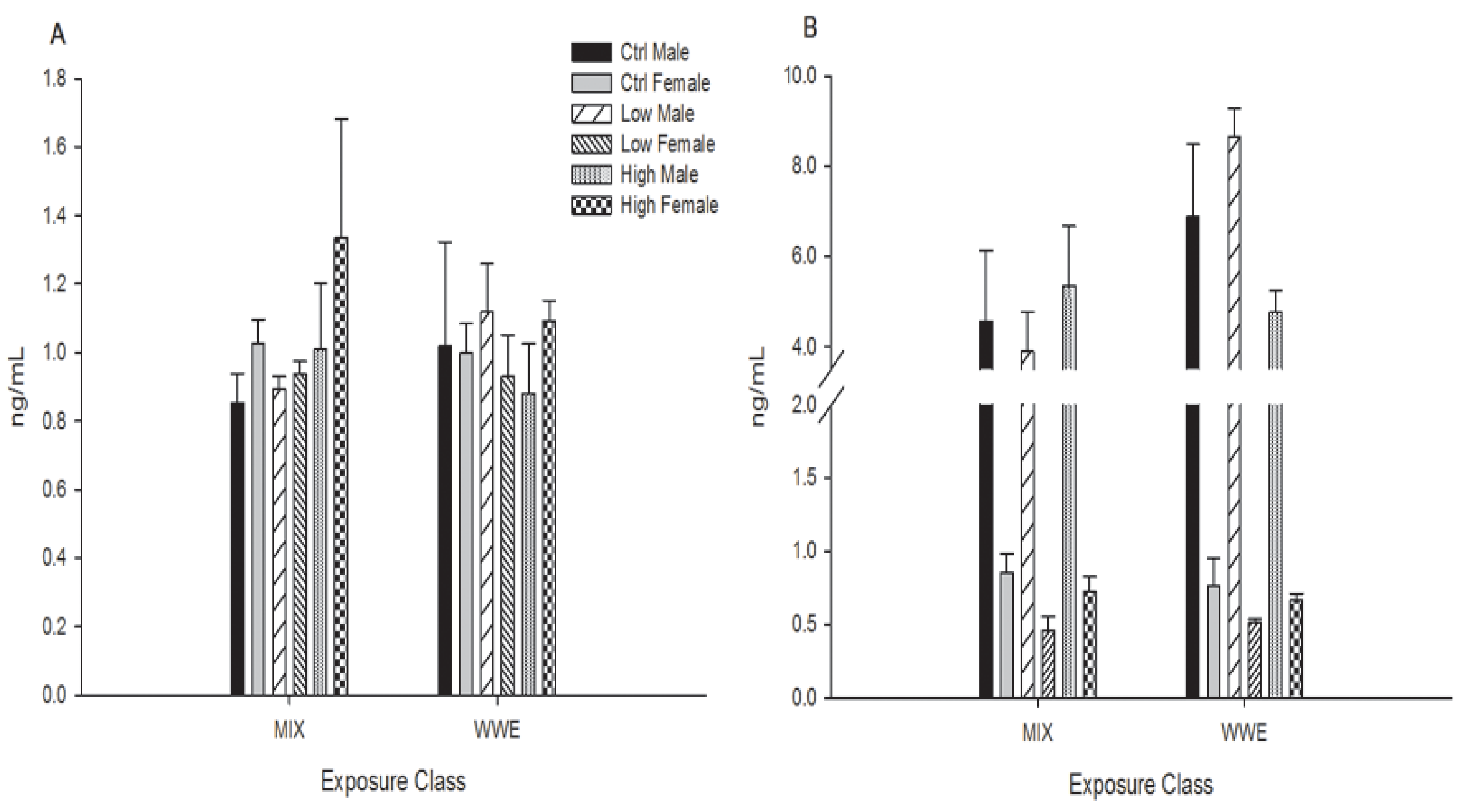
**Supplemental Table S7: Incidence and severity of histopathological damage in the kidney of zebrafish exposed to a mixture of pharmaceuticals (MIX) and wastewater effluent (WWE)**. Adult fish were exposed for 6 weeks at 0 (control), 0.5 (low) and 10 (high) $\mu g\ L^{-1}$ to a mixture of acetaminophen, carbamazepine, gemfibrozil and venlafaxine (MIX) and to 0 (control), 5% (low) and 25% (high) waste-water effluent (WWE).Whole zebrafish were embedded, serially sectioned at 5µm, and stained with hematoxylin and eosin prior to screening under 40X magnification and standard light microscopy. Control fish from all experiments were not statistically different in the incidence of histopathology or mean score. Histopathological scores are adapted from Bernet et al. (1999). Intensity of damage was scored on a 5 point scale with 0/1 representing no or very minimal damage to the kidney tubule, 2 – moderate loss of cytoplasm within the kidney tubule and nuclei vacuolization, 3 – very limited cytoplasm within the kidney tubule with noncentral nuclei positioning and 4 – complete loss of native structure and staining integrity; see materials and methods for details. All exposures showed a statistically significant increase in the incidence of pathology and the mean histopathological score, when compared to controls; $P \leq 0.05$.

| **Sex** | **Treatment** | **N** | **Score** | | | | | **Incidence** |
|---|---|---|---|---|---|---|---|---|
| | | | **0** | **1** | **2** | **3** | **4** | |
| ***Males*** | Mix Ctrl | 23 | 20 | 3 | 0 | 0 | 0 | 0.13 |
| | Mix Low | 34 | 1 | 0 | 0 | 16 | 17 | 0.97 |
| | Mix High | 30 | 0 | 0 | 2 | 10 | 18 | 1.00 |
| | WWE Ctrl | 26 | 21 | 5 | 0 | 0 | 0 | 0.19 |
| | WWE Low | 24 | 1 | 0 | 2 | 7 | 14 | 0.96 |
| | WWE High | 22 | 2 | 0 | 1 | 10 | 9 | 0.91 |
| ***Females*** | Mix Ctrl | 27 | 26 | 0 | 1 | 0 | 0 | 0.04 |
| | Mix Low | 22 | 1 | 0 | 0 | 20 | 1 | 0.95 |
| | Mix High | 23 | 2 | 0 | 9 | 11 | 1 | 0.91 |
| | WWE Ctrl | 26 | 0 | 0 | 0 | 0 | 0 | 0.0 |
| | WWE Low | 23 | 6 | 1 | 4 | 10 | 2 | 0.74 |
| | WWE High | 25 | 2 | 1 | 7 | 14 | 1 | 0.92 |

**Supplemental Table S8: Incidence and severity of histopathological damage in the ovaries of female zebrafish exposed to a mixture of pharmaceuticals (MIX) and wastewater effluent (WWE)**. Adult fish were exposed for 6 weeks at 0 (control), 0.5 (low) and 10 (high) μg $L^{-1}$ to a mixture of acetaminophen, carbamazepine, gemfibrozil and venlafaxine (MIX) and to 0 (control), 5% (low) and 25% (high) waste-water effluent (WWE).Whole zebrafish were embedded, serially sectioned at 5μm, and stained with hematoxylin and eosin prior to screening under 40X magnification and standard light microscopy. Control fish from all experiments were not statistically different in the incidence of histopathology or mean score Histopathological scores are adapted from Bernet et al. (1999). Intensity of damage was scored on a 3 point scale. Females scored 0 display no or very minimal damage to the gonad, 1 – an increase in stromal tissue within the oocyte and 2 –smaller mature oocytes with vitellogenin degradation; see materials and methods for details. All exposures showed a statistically significant increase in the incidence of histopathology and mean histopathological score, when compared to controls; $P \leq 0.05$.

| **Treatment** | **N** | **Score** | | | **Incidence** |
|---|---|---|---|---|---|
| | | **0** | **1** | **2** | |
| **Mix Ctrl** | 27 | 26 | 1 | 0 | 0.04 |
| **Mix Low** | 22 | 6 | 12 | 4 | 0.73 |
| **Mix High** | 23 | 13 | 10 | 0 | 0.44 |
| **WWE Ctrl** | 26 | 22 | 3 | 1 | 0.15 |
| **WWE Low** | 23 | 11 | 9 | 3 | 0.52 |
| **WWE High** | 25 | 7 | 15 | 3 | 0.72 |

**Supplemental Figure S1: Mean (N=3) plasma concentrations of estradiol and 11-ketotestosterone in adult male and female zebrafish after 6 weeks exposure to a pharmaceutical mix (MIX) and to wastewater effluent (WWE)**. After 6 weeks of exposure to 0 (ctrl), 0.5 (low) and 10 (high) µg $L^{-1}$of a mixture of pharmaceuticals or 0 (ctrl), 5 (low) and 25 (high) % effluent, zebrafish were euthanatized and blood was collected from the caudal vein. Plasma concentrations of A) Estradiol and B) 11-Ketotestosterone are expressed as ng $mL^{-1}$.


A
Ctrl Male
Ctrl Female
Low Male
Low Female
High Male
High Female
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ng/mL
MIX
WWE
Exposure Class
B
10.0
8.0
6.0
4.0
2.0
1.5
1.0
0.5
0.0
ng/mL
MIX
WWE
Exposure Class

**- THE END -**