PARENTAL EFFECTS ON SEXUAL DIFFERENTIATION IN ZEBRAFISH
THE EFFECTS OF PARENTAL CARBAMAZEPINE AND GEMFIBROZIL EXPOSURE ON SEXUAL DIFFERENTIATION IN ZEBRAFISH (Danio rerio)

By:

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

McMaster University
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TITLE: The Effects of Parental Carbamazepine and Gemfibrozil Exposure on Sexual Differentiation in Zebrafish (Danio rerio)

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LAY ABSTRACT

Parental exposure to the environmentally-relevant pharmaceuticals carbamazepine or gemfibrozil led to male-biased sex ratios in adult offspring of zebrafish (*Danio rerio*), a common model organism. The development of the gonads in juveniles was investigated to determine how this process was impacted. Predominately, paternal exposure was found to result in a faster development of the testes and male-biased sex ratios. Interestingly, sex ratios in juveniles did not always reflect those in adults, suggesting a sex reversal may have occurred in adulthood. This study demonstrates the ability of pharmaceuticals to alter gonad development in offspring of exposed parents.
ABSTRACT

Endocrine-disrupting compounds (EDCs) interfere with the physiology of hormone systems. Traditionally, steroidogenic pharmaceuticals have been studied as EDCs however there has been growing evidence that non-steroidogenic pharmaceuticals can alter sex steroid levels and impair reproductive functions in fish. This is of concern as pharmaceuticals are detected in surface waters at the ng L\(^{-1}\) to µg L\(^{-1}\) range. Zebrafish (Danio rerio) were exposed to 10 µg L\(^{-1}\) of the pharmaceuticals carbamazepine and gemfibrozil for 6 weeks. Male-biased sex ratios were observed in the sexually mature offspring after paternal exposure, suggesting that sexual differentiation may be impacted in juveniles. Currently, the ability of pharmaceuticals to interfere with sexual differentiation of parentally exposed offspring is unknown. This thesis examined the gonad histology of juvenile zebrafish to understand how sexual differentiation was affected in the offspring of exposed parents. Paternal, but not maternal, exposure to carbamazepine resulted in a significantly faster sexual differentiation of the gonads and led to a male-biased sex ratio; these effects were not observed when both parents were exposed. Combined paternal and maternal exposure to gemfibrozil resulted in significantly faster sexual differentiation and paternal, but not maternal, exposure to gemfibrozil led to male-biased sex ratios. Interestingly, sex ratios observed in the juveniles did not always reflect those found in the same lineage at sexual maturity, suggesting a sex reversal, including a male to female transition, occurred past the juvenile sexual differentiation period in some fish. This thesis demonstrates that pharmaceuticals have the ability to disrupt sexual differentiation in the F\(_1\) offspring of exposed parents and that paternal exposure is most relevant for offspring effects.
ACKNOWLEDGMENTS

There are many people (and animals) I need to send a special thank-you to who have helped to make this thesis a success. Now let’s see if I can make it through everyone before the music kicks in...

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<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>µg g⁻¹</td>
<td>Microgram per Gram</td>
</tr>
<tr>
<td>µg L⁻¹</td>
<td>Microgram per Litre</td>
</tr>
<tr>
<td>µg mL⁻¹</td>
<td>Microgram per Millilitre</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometers</td>
</tr>
<tr>
<td>µS</td>
<td>Microsiemens</td>
</tr>
<tr>
<td>amh</td>
<td>Anti-Müllerian Hormone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ar</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ca</td>
<td>Cortical Alveolar Growth Oocyte</td>
</tr>
<tr>
<td>CBZ</td>
<td>Carbamazepine</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>CTL</td>
<td>Control</td>
</tr>
<tr>
<td>cyp11b</td>
<td>Cytochrome P450, Family 11, Subfamily B; 11β-Hydroxylase</td>
</tr>
<tr>
<td>cyp19a1a</td>
<td>Cytochrome P450, Family 19, Subfamily A, Polypeptide 1a; Aromatase (gonadal derived gene)</td>
</tr>
<tr>
<td>cyp19a1b</td>
<td>Cytochrome P450, Family 19, Subfamily B, Polypeptide 1b; Aromatase (brain derived gene)</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>dmrt1</td>
<td>Doublesex and Mab-3 Related Transcription Factor 1</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>dpf</td>
<td>Days Post Fertilization</td>
</tr>
<tr>
<td>dph</td>
<td>Days Post Hatch</td>
</tr>
<tr>
<td>EDCs</td>
<td>Endocrine-disrupting Compounds</td>
</tr>
<tr>
<td>EE2</td>
<td>17α-Ethinylestradiol</td>
</tr>
<tr>
<td>F</td>
<td>Female</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>F₁</td>
<td>First Filial Generation</td>
</tr>
<tr>
<td>F₂</td>
<td>Second Filial Generation</td>
</tr>
<tr>
<td>F₃</td>
<td>Third Filial Generation</td>
</tr>
<tr>
<td>ff₁b</td>
<td>Fushi Tarazu Factor 1b</td>
</tr>
<tr>
<td>ff₁d</td>
<td>Fushi Tarazu Factor 1d</td>
</tr>
<tr>
<td>GEM</td>
<td>Gemfibrozil</td>
</tr>
<tr>
<td>GSI</td>
<td>Gonadosomatic Index</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>Kᵢₐw</td>
<td>Octanol/Water Partition Coefficient</td>
</tr>
<tr>
<td>Lₜ</td>
<td>Total Length</td>
</tr>
<tr>
<td>M</td>
<td>Male</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>MT</td>
<td>$17\alpha$-Methyltestosterone</td>
</tr>
<tr>
<td>mg kg⁻¹</td>
<td>Milligrams per Kilogram</td>
</tr>
<tr>
<td>mg L⁻¹</td>
<td>Milligrams per Litre</td>
</tr>
<tr>
<td>n</td>
<td>Nucleus</td>
</tr>
<tr>
<td>N/A</td>
<td>Not Applicable</td>
</tr>
<tr>
<td>ng L⁻¹</td>
<td>Nanograms per Litre</td>
</tr>
<tr>
<td>N/O</td>
<td>Not Observed</td>
</tr>
<tr>
<td>nr0b1</td>
<td>Nuclear Receptor Subfamily 0, Group B, Member 1</td>
</tr>
<tr>
<td>o</td>
<td>Ooplasm</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for Economic Co-operation and Development</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>pH</td>
<td>Measure of Acidity or Basicity/ -log[H⁺]</td>
</tr>
<tr>
<td>po</td>
<td>Perinucleolar Oocytes</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisomal Proliferator-activated Receptor Alpha</td>
</tr>
<tr>
<td>sc</td>
<td>Spermatocytes</td>
</tr>
<tr>
<td>sd</td>
<td>Spermatids</td>
</tr>
<tr>
<td>sg</td>
<td>Spermatogonia</td>
</tr>
<tr>
<td>sox9a</td>
<td>SRY (sex determining region Y)-box 9a</td>
</tr>
<tr>
<td>sz</td>
<td>Spermatozoa</td>
</tr>
<tr>
<td>TBT</td>
<td>Tributyltin</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-Tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>tp53</td>
<td>Tumour Protein 53</td>
</tr>
<tr>
<td>wt1a</td>
<td>Wilms Tumour 1a</td>
</tr>
<tr>
<td>WWTP</td>
<td>Wastewater Treatment Plant</td>
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DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis is organized in a standard format and consists of three chapters. Chapter 1 comprises an introduction to the broader themes of this thesis including endocrine disruption and an examination of pharmaceuticals as endocrine disruptors in fish. Details are provided on the morphology of the gonads and sexual differentiation in zebrafish. The objectives are outlined. Chapter 2 comprises a manuscript focused on the effects of parental pharmaceutical exposure on sexual differentiation in zebrafish. Chapter 3 examines the research in a broader context including potential mechanisms for parental effects and the relevance of the results, as well as establishes directions for future research.

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: THE EFFECTS OF PARENTAL CARBAMAZEPINE AND GEMFIBROZIL EXPOSURE ON SEXUAL DIFFERENTIATION IN ZEBRAFISH (Danio Rerio)

Authors: Kristine M. Hammill, Shamaila Fraz and Joanna Y. Wilson

Comments: K.M.H. conducted this study and wrote the manuscript under the supervision of J.Y.W. S.F. conducted the exposure and sampled the offspring. All authors provided editorial comments.

CHAPTER 3: GENERAL DISCUSSION
CHAPTER 1
GENERAL INTRODUCTION

1.1 Endocrine Disruption

Endocrine-disrupting compounds (EDCs) can be defined as “natural or man-made substances that disrupt function, levels, and distribution of endogenous hormones of exposed organisms by either mimicking or antagonizing the actions of hormones, or by modulating hormone synthesis or metabolism” (Segner 2009). EDCs are of concern as hormones regulate physiological processes in both vertebrates and invertebrates (Edelman 1975; LaFont 2000; Nandi 1967), and organisms have evolved a sensitivity to these chemical signals in order to maintain internal homeostasis (Cheek et al. 1998). As such, EDCs have the potential to disturb the state of homeostasis at low concentrations. Aside from the obvious potential for hormonal interactions in the gonads and brain, there are many other organ systems (e.g. skeleton, immune, renal) that are all potential targets of EDCs (Colborn et al. 1993). In particular, an emphasis has been placed on the impacts of EDCs on sex determination, sexual differentiation and sexual development, due to their direct link with reproduction.

Vertebrate sex determination is “the genetic [and/or] environmental process by which the sex of an individual is established” (Penman and Piferrer 2008), while sexual differentiation is “the process by which an undifferentiated gonad is transformed into an ovary or testis” (Penman and Piferrer 2008). Sexual differentiation occurs through molecular, genetic and physiological mechanisms given the genotype and environment of
an organism (Piferrer and Guiguen 2008). In vertebrates, sex-related functions, including sexual differentiation, are largely controlled by estrogens (mainly 17β-estradiol, but also estrone and estriol) and androgens (mainly testosterone and 5α-dihydrotestosterone, or 11-ketotestosterone in fish; Kime 1993; Tyler et al. 1998). There has even been increasing evidence for the presence and function of these sex steroids in several invertebrates (Janer and Porte 2007; Keay and Thornton 2009).

Endocrine disruption has been observed in wild populations of animals including molluscs, fish, reptiles, birds and mammals (for review see Tyler et al. 1998), with several well-documented case studies. In one notable case, a dramatic decline in an alligator population was observed during the 1980s in Lake Apopka in Florida, which was attributed to a chemical spill of dicofol, DDT and its metabolites and sulfuric acid (Guillette et al. 1994). Juvenile alligators had significantly altered plasma steroid concentrations (almost two times greater 17β-estradiol levels in females and more than three times lower testosterone levels in males as compared to animals from the control site), which was accompanied by an abnormal morphology of the gonads (Guillette et al. 1994).

Tributyltin (TBT), an antifouling agent commonly used on boat halls, has been linked to the conditions of imposex (male sex organs superimposed on female sex organs) and intersex (female sex organs modified towards male structure followed by supplantation of male sex organs) in gastropod molluscs (sea snails; for reviews see Matthiessen and Gibbs 1998; Oehlmann et al. 2007). While both conditions can impede or prevent reproduction, the former has been associated with population declines or
disappearances (Evans et al. 1996; Matthiessen and Gibbs 1998; Oehlmann et al. 2007). Restrictions have been placed on TBT-based paints (Champ 2000; Terlizzi et al. 2001) and the International Maritime Organization implemented a complete prohibition on TBT in 2008 (www.imo.org/en/OurWork/Environment/Anti-foulingSystems).

Perhaps one of the most commonly known connections between industrial activities and endocrine disruption is that of pulp and paper mill effluent and its effects on fish. Pulp and paper mill effluent has been documented to reduce gonadal development, decrease serum $17\beta$-estradiol and testosterone concentrations, lower fecundity with age in females, suppress secondary sexual characteristics in males and partially or completely masculinize females living downstream from pulp processing plants (Bortone et al. 1989; Bortone and Cody 1999; Howell et al. 1980; Howell and Denton 1989; Larsson et al. 2000; Munkittrick et al. 1991, 1992,1998; Parks et al. 2001). It should be noted that changes in the processing method at pulp and paper mills can mitigate the effects observed to varying degrees (Munkittrick et al. 1998).

Additionally, wastewater treatment plant (WWTP) effluent has been implicated in endocrine disruption, including female-biased sex ratios and the presence of an egg-related protein (vitellogenin) in males (Jobling et al. 1998; Vajda et al. 2008). Intersex (presence of eggs and/or additional ovarian tissue in testes) has been found to occur in wild populations of riverine fish throughout the UK downstream from WWTP effluent discharge (Jobling et al. 1998; Nolan et al. 2001).
Observations of endocrine disruption in the wild have not been limited to chemical or effluent discharges. In two separate estuarine populations of Atlantic croaker (*Micropogonias undulatus*) seasonal hypoxia has been shown to significantly reduce plasma steroid concentrations, suppress ovarian and testicular growth, impair gametogenesis, reduce fecundity, and ultimately in one case, disturb reproduction in a 42 km² region (Thomas et al. 2006, 2007).

As the above cases highlight, endocrine disruption has become widely documented. Today the question has progressed from whether endocrine disruption occurs due to exposure to contaminants, to more broadly encompass the questions of at what concentration does it occur, what is the mechanism of action, or will there be an adverse response at the population level (Guillette 2006)? Much attention has been given to seeking the answer to these questions with fish, given their economic and recreational significance (for review see Mills and Chichester 2005).

1.2 Pharmaceuticals as Endocrine Disruptors in Fish

Pharmaceuticals can be found in surface waters in the ng L⁻¹ to µg L⁻¹ range (Ashton et al. 2004; Boyd et al. 2004; Brun et al. 2006; da Silva et al. 2011; Kolpin et al. 2002; Koné et al. 2013; Kuch and Ballschmiter 2001; Lissemore et al. 2006; Metcalfe et al. 2003; Peng et al. 2008; Stumpf et al. 1999; Zhang et al. 2007). This is largely due to human consumption of pharmaceuticals. After human consumption, pharmaceuticals are excreted in urine or feces as their parent compound or metabolite(s) and enter
wastewater (Fent et al. 2006; Halling-Sørensen et al. 1998). Alternative sources can introduce pharmaceuticals into the environment, including improper disposal (i.e. down the drain), hospital and manufacturers wastewater, landfill leachate, veterinary applications with livestock and aquaculture, as well as from the spreading of manure or biosolids onto agricultural fields followed by runoff into surface water (Fent et al. 2006; Halling-Sørensen et al. 1998; Holm et al. 1995). While those pharmaceuticals that enter wastewater are subject to treatment, pharmaceuticals are not readily or fully degraded in wastewater treatment plants, and the parent compound, their metabolite(s), and/or their transformation product(s) are discharged into the environment via wastewater effluent (Fent et al. 2006; Halling-Sørensen et al. 1998). Since pharmaceuticals are designed to exert a biological effect at low doses, they have the potential to harm aquatic wildlife, either through their intended mode of action or an unknown mechanism (for review see Fent et al. 2006).

To determine if pharmaceuticals have endocrine disrupting and related effects in fish, common endpoints such as plasma steroid concentrations, sexual differentiation, gonad morphology, gonadosomatic index (GSI; the gonad mass as a proportion of the total body mass), gamete development, sperm parameters (counts, viability counts, motility, velocity etc.), sex ratio, and secondary sexual characteristics, have been utilized. Changes in enzymes in the steroidogenic pathway, for example, aromatase, the enzyme that converts androgens into estrogens, have also been examined (Scholz and Gutzeit 2000). In addition, reproductive parameters are often examined due to their direct link with the
endocrine system and their ability to manifest in population level effects. One particularly important endpoint, often used as a biomarker for the presence of estrogenic compounds in the environment, is the detection of vitellogenin in males. Vitellogenin is a protein produced in the liver of female fish primarily under the control of 17β-estradiol. Vitellogenin is transported to the ovary, where it is taken into oocytes via endocytosis and processed into yolk proteins. This entire process is referred to as vitellogenesis (Sumpter and Jobling 1995). Exposure to estrogenic substances can cause the production of vitellogenin in males (where it is normally not detected), as well as increased or prolonged induction in females (Kidd et al. 2007; Purdom et al. 1994; Sumpter and Jobling 1995). Changes in vitellogenin can be detected through gene expression or plasma protein concentrations.

By far the most studied pharmaceutical in terms of endocrine disruption is 17α-ethinylestradiol (EE2) which is widely used in oral contraceptive pills (Frye 2006). Given that it is designed to mimic the effects of 17β-estradiol in humans (Frye 2006; Rivera et al. 1999), it has the same potential for other vertebrates. In particular, the impact of exposure during early life stages has been emphasized as sexual differentiation occurs in fish post hatch (Maack and Segner 2003; Nakamura 1984; Nakamura et al. 1998; van Aerle et al. 2004) and is thought to be a heightened time of sensitivity to EDCs. The specific responses are dependent on species, dose, duration as well as timing of the exposure.
In male rainbow trout (*Oncorhynchus mykiss*) undergoing sexual maturation, a dose of 2 ng L\(^{-1}\) of EE2 over a 3 week period caused a 60% inhibition of testicular growth and altered the proportion of germ cell stages present in the testis (Jobling et al. 1996). Furthermore, doses of only 0.1 ng L\(^{-1}\) and above have been shown to cause significant elevation of plasma vitellogenin after 10 days of exposure in male trout (Purdom et al. 1994)

Juvenile Japanese medaka (*Oryzias latipes*) exposed to EE2 for 2 months had a significant decrease in the GSI and egg production in females at doses as low as 10 ng L\(^{-1}\), while aromatase (normally not expressed in testes) was detected in the testis of males exposed to 10 ng L\(^{-1}\) (Scholz and Gutzeit 2000). Increased vitellogenin has been detected in whole body samples following exposure to 100 ng L\(^{-1}\) of EE2 from 0 to 60 days post hatch (dph; Örn et al. 2006). Furthermore, intersex has been observed in juvenile medaka at doses as low as 10 ng L\(^{-1}\), as well as female-biased sex ratios, including one case of complete sex reversal after exposure to 100 ng L\(^{-1}\) of EE2 for 2 months, even following a 6-week recovery period (Örn et al. 2006; Scholz and Gutzeit 2000).

In juvenile zebrafish (*Danio rerio*), following exposure to EE2 for various periods between 0 to 90 dph, vitellogenin induction, female-biased sex ratios and increased mortality have been observed at doses as low as 2 ng L\(^{-1}\), 1 ng L\(^{-1}\), and 2 ng L\(^{-1}\), respectively (Fenske et al. 2005; Örn et al. 2003, 2006; Xu et al. 2008). Complete feminization has been observed at doses as low as 2 ng L\(^{-1}\) (Andersen et al. 2003; Fenske et al. 2005; Örn et al.
2003, 2006; Xu et al. 2008) and 100% mortality has been documented at a dose of 100 ng L\(^{-1}\) (Örn et al. 2006). Exposure for even a limited of period time between 0 to 60 dph can be harmful, and 20 to 60 dph, during which sexual differentiation occurs, has proven to be an especially critical window for zebrafish (Andersen et al. 2003). Likewise, a full-life cycle exposure to 3 ng L\(^{-1}\) of EE2 resulted in elevated vitellogenin concentrations and 100% feminization (Fenske et al. 2005). Studies suggest that with a recovery period (i.e. rearing in clean water), sex ratio has the ability to recover to varying degrees, even after a full-life cycle exposure, however reproduction may remain impaired and vitellogenin induction in females can last into adulthood (Fenske et al. 2005; Xu et al. 2008). Furthermore, significant testicular abnormalities have been observed even after a 3 month recovery period including malformations of the sperm duct, an altered proportion of the germ cell types and a reduced number of spermatozoa in male zebrafish exposed to 2 and 10 ng L\(^{-1}\) of EE2 from 0 to 90 dph (Xu et al. 2008).

In fathead minnow (\textit{Pimephales promelas}) various exposure periods between fertilization to 20 dph have all been found to be harmful, with doses of 10 ng L \(^{-1}\) of EE2 causing induction of vitellogenin and disruption of duct development (a sign of feminization) in males. Furthermore, exposure to 10 ng L\(^{-1}\) between 10-15 dph and from fertilization to 20 dph caused a decrease in the number of spermatozoa in male testes (van Aerle et al. 2002). A full-life cycle study suggests that impacts can worsen with longer exposures. Länge et al. (2001) saw an increase in intersex starting at 1 ng L\(^{-1}\) at 56 dph, while by 172 dph a female-skewed sex ratio was observed at a dose of 1 ng L\(^{-1}\) and full
feminization starting at doses of 4 ng L⁻¹. Gonad morphology was also impacted by a full-life cycle exposure, with mild degeneration of the testes starting at 1 ng L⁻¹ and progressive degeneration of the ovaries starting at 16 ng L⁻¹ (Länge et al. 2001).

Impacts of EE2 appear to uphold in the field. In a whole lake study it was shown that 5–6 ng L⁻¹ of EE2 (added 3 times weekly in the open-water season over 3 years) led to the feminization of male fathead minnows (production of vitellogenin mRNA and protein), a continued production of vitellogenin in females post-breeding season, impacts on gonadal development including intersex in males and altered oogenesis in females, and as a result nearly caused the extirpation of the fathead minnow population (Kidd et al. 2007).

In surface waters EE2 ranges from being undetectable to concentrations as high as 831 ng L⁻¹ (Kolpin et al. 2002; Kuch and Ballschmiter 2001; Peng et al. 2008), giving reason for environmental concern. It should be noted that the most active natural estrogen, 17β-estradiol, can also be found in surface waters ranging from below detection to concentrations as high as 200 ng L⁻¹ (Boyd et al. 2004; Kolpin et al. 2002; Kuch and Ballschmiter 2001; Peng et al. 2008; Zhang et al. 2007), likely as a result of excretion in urine from women. 17β-estradiol can impact fish in similar ways to EE2 (Metcalf et al. 2001).

In contrast, 17α-methyltestosterone (MT) is a synthetic hormone that has been used to treat males with testosterone deficiency (Bhasin et al. 2010; Buvat et al. 2013)
and is considered a potent androgen receptor agonist. In aquaculture it has been applied to control sex determination and promote masculinization of fish (Feist et al. 1995; Vanden Hurk et al. 1989). In zebrafish exposed to MT from 20 to 60 dph, male-biased sex ratios were observed starting at 26 ng L\(^{-1}\), with complete masculinization observed at doses as low as 100 ng L\(^{-1}\) and cases of intersex observed at higher doses of 260 and 1000 ng L\(^{-1}\) (Örn 2003). The latter is concurrent with the ability of MT to be converted by aromatase to 17β-estradiol (indirectly shown by Hori et al. 1979 via its ability to induce vitellogenesis) and actually cause feminization at higher doses. In zebrafish exposed to a higher dose of 10 µg L\(^{-1}\) of MT from 35 to 71 days post fertilization (dpf), 100% feminization was observed, while \(cyp19a1a\) (the gonadal derived gene for aromatase) mRNA expression was suppressed and \(cyp19a1b\) (the brain derived gene for aromatase) mRNA expression was increased (Fenske and Segner 2004). Additionally, MT was shown to alter vitellogenin concentrations during the period of sexual differentiation in zebrafish starting at 100 ng L\(^{-1}\) (Örn et al. 2003). At this time there have been no reports on the concentration of MT in surface waters.

In addition to synthetic hormones, pharmaceuticals from an array of classes have been linked to endocrine disruption and reproductive impairment in fish. Fadrozole, a second generation nonsteroidal aromatase inhibitor, is used in the treatment of postmenopausal hormone-responsive breast cancer (Miller 2003). In adult fathead minnows exposed to fadrozole for 21 days numerous effects have been observed. Plasma steroid concentrations have been impacted with decreases in plasma 17β-estradiol in
females and increases in testosterone and 11-ketotestosterone observed in males at doses as low as 10 µg L\(^{-1}\). Vitellogenin concentrations have been found to decrease in females and increase in males, at doses as low as 2 µg L\(^{-1}\) and 25 µg L\(^{-1}\), respectively. In males, enhanced testis growth (a higher GSI) including enlargement of lumina and seminiferous tubules has been observed at doses as low as 10 µg L\(^{-1}\). In females, an increase in the number of pre-ovulatory follicles and atretic follicles has been observed, as well as less mature ovaries and inhibited ovarian growth (a lower GSI) at doses as low 2, 10 and 25 µg L\(^{-1}\) respectively (Ankley et al. 2002; Panter et al. 2004). Furthermore, a decrease in fecundity has been observed at doses as low as 2 µg L\(^{-1}\) (Ankley et al. 2002). Consistent with the mechanism of action for fadrozole, brain aromatase activity was found to be significantly reduced in both sexes (Ankley et al. 2002). In nile tilapia (\textit{Oreochromis niloticus}) exposed during the period of sexual differentiation (7 to 37 dph), doses between 200–500 mg kg\(^{-1}\) caused >90% masculinization (Kwon et al. 2000). Likewise, in zebrafish fed 500 µg g\(^{-1}\) twice per day during the period of differentiation (35 to 71 dpf), 100% masculinization and suppressed mRNA expression of \textit{cyp19a1a} were observed (Fenske and Segner 2004). No sex reversal was observed after rearing the treated fish under control conditions until 161 dpf, however at this time point 14/22 fish showed \textit{cyp19a1a} mRNA levels similar to those detected in control testes, while in the other eight fish levels reflected those found in control ovaries (Fenske and Segner 2004). Likewise to MT, there have been no reports on whether fadrozole has been detected in surface waters.
Fluoxetine is used as an antidepressant that acts as a selective serotonin reuptake inhibitor (Sulser et al. 1978) and has been linked to endocrine disruption in several fish species. In adult Japanese medaka exposure to fluoxetine for 4 weeks was found to increase plasma 17β-estradiol concentrations at doses of 0.1 and 0.5 µg L⁻¹ (Foran et al. 2004). In sexually mature female zebrafish exposure to 32 µg L⁻¹ of fluoxetine for 7 days was found to cause a significant reduction (4.5 fold) in the average number of eggs spawned. This coincided with a decrease in ovarian levels of 17β-estradiol as well as a reduction in ovarian aromatase, follicle stimulating hormone receptor and luteinizing hormone receptor gene expression (Lister et al. 2009). In sexually mature male goldfish exposure to fluoxetine for 14 days was found to impact the reproductive physiology. These impacts included a significant decrease in basal milt volume at 54 µg L⁻¹, pheromone-stimulated milt volume at 0.54 and 54 µg L⁻¹, as well as decreases in basal and pheromone-stimulated testosterone levels at 54 µg L⁻¹. Additionally, a significant concentration dependent decrease in follicle stimulating hormone was observed in pheromone-stimulated fish as well as a concentration dependent increase of 17β-estradiol levels (Mennigen et al. 2010). Fluoxetine can be found in surface waters at concentrations up to 0.141 µg L⁻¹ (Kolpin et al. 2002; Koné et al. 2013; Metcalfe et al. 2003), indicating one study observed an impact at an environmentally relevant concentration.

Propranolol, a β-adrenergic blocker, has been linked to endocrine disruption in Japanese medaka. Following a 2-week exposure to propranolol, males were found to have
a significantly decreased plasma testosterone and increased plasma 17β-estradiol concentrations at doses as low as 0.5 µg L\(^{-1}\), while females had significantly increased plasma 17β-estradiol concentrations at doses as low 100 µg L\(^{-1}\) (Huggett et al. 2002). No changes in reproduction were observed, but in a follow-up 4-week exposure period the number of eggs produced was significantly reduced at 0.5 and 1 µg L\(^{-1}\) (Huggett et al. 2002). Propranolol has been detected in surface waters at concentrations ranging up to 0.215 µg L\(^{-1}\) (Ashton et al. 2004; Koné et al. 2013), concentrations below which effects have been observed in laboratory exposures.

Several analgesics, including acetaminophen and the nonsteroidal anti-inflammatory drugs ibuprofen and indomethacin, have been linked to endocrine disruption and reproductive effects in fish. These pharmaceuticals act through the inhibition of cyclooxygenase activity (Hinz et al. 2008; Mitchell et al. 1993; Vane and Botting 1998). In teleosts, the cyclooxygenase pathway is involved with oocyte maturation and ovulation (Patiño and Sullivan 2002). Adult Japanese medaka exposed to ibuprofen for 6 weeks were found to have an altered reproductive pattern, with increasing concentrations (significant at 100 µg L\(^{-1}\)) of ibuprofen causing a decreased frequency of egg production, but an increase in egg production when spawning events occurred (Flippin et al. 2007). In zebrafish exposed to indomethacin for 16 days, a significant reduction in clutch size was observed as well as a large reduction in spawning rate at a dose of 100 µg L\(^{-1}\) (Lister and Van Der Kraak 2008). In vitro, indomethacin was shown to significantly reduce the rate of zebrafish oocyte maturation at 10 µg mL\(^{-1}\) (Lister and Van
Der Kraak 2008). Similarly, in zebrafish exposed to acetaminophen over a 6-week period, a reduced embryo production was observed at a dose of 10 µg L\(^{-1}\) while an increase in apoptotic ovarian follicles was observed at ≥0.5 µg L\(^{-1}\) (Galus et al. 2013). In surface waters, ibuprofen, indomethacin and acetaminophen range from not detected to concentrations as high as 6.4 µg L\(^{-1}\), 0.15 µg L\(^{-1}\), and 10 µg L\(^{-1}\) respectively, with the latter being found within the range of effect concentrations in labs (Ashton et al. 2004; Boyd et al. 2004; Brun et al. 2006; da Silva et al. 2011; Kolpin et al. 2002; Koné et al. 2013; Metcalfe et al. 2003; Peng et al. 2008; Zhang et al. 2007).

The fibrate lipid regulator gemfibrozil and metabolite, clofibric acid, have been implicated in endocrine disruption and related effects. In male goldfish (\textit{Carassius auratus}) exposure to 1.5 µg L\(^{-1}\) of gemfibrozil (GEM) for 14 days was shown to reduce plasma testosterone by 50% (Mimeault et al. 2005). In adult zebrafish, exposure to GEM for 6 weeks was found to induce histopathological changes to the ovary starting at 0.5 µg L\(^{-1}\) with a reduced maturity of the oocytes apparent at 10 µg L\(^{-1}\). This coincided with a reduced fecundity over the 6-week exposure at doses of 0.5 and 10 µg L\(^{-1}\) (Galus et al. 2013). In the \(F_1\) generation of zebrafish whose parents were exposed to 10 µg L\(^{-1}\) of GEM for 4 weeks, reduced fecundity as well as altered courtship, sperm velocity and sperm morphology has been observed (Galus et al. 2014). Clofibric acid, the biologically active metabolite of the lipid regulator clofibrate, has been shown to impact spermatogenesis in fathead minnow after 21 days including a reduction in sperm count starting at doses of 10 µg L\(^{-1}\) and a reduction in measures of sperm motility at 1 mg L\(^{-1}\). Furthermore, a trend of
reduced plasma androgen concentrations was noted in the same study (Runnalls et al. 2007). GEM and clofibric acid range in surface waters from not detected to concentrations as high as 0.79 µg L⁻¹ and 0.248 µg L⁻¹, respectively (Brun et al. 2006; da Silva et al. 2011; Kolpin et al. 2002; Koné et al. 2013; Metcalfe et al. 2003; Peng et al. 2008; Stumpf et al. 1999), indicating effects of GEM have been observed at an environmentally relevant concentration.

Carbamazepine (CBZ), primarily used as an antiepileptic and mood stabilizer, has been found to have numerous impacts on zebrafish. Exposure to CBZ for 6-weeks caused a 9.5-fold and 2.2-fold decrease in plasma 11-ketotestosterone levels in males and females, respectively, at 0.5 µg L⁻¹ (Galus et al. 2013). Furthermore, histopathological changes to the ovary were observed at doses as low as 0.5 µg L⁻¹, with a decreased maturity of the oocytes noted at 10 µg L⁻¹ (Galus et al. 2013). Over the course of the exposure CBZ was found to reduce fecundity in adult zebrafish at 0.5 and 10 µg L⁻¹ (Galus et al. 2013). In the F₁ generation of zebrafish whose parents were exposed to CBZ at 10 µg L⁻¹ for 4 weeks reduced fecundity as well as altered courtship, sperm velocity and sperm morphology has been observed (Galus et al. 2014). In the common carp (Cyprino carpio L.), CBZ has been shown to significantly impact spermatozoa at doses of 2.0 and 20 mg L⁻¹ after a 2 h incubation, including decreased motility and velocity, and induced oxidative stress (Li et al. 2010). CBZ has been found in surface waters at concentrations as high as 0.7 µg L⁻¹ (Benotti and Brownawell 2007; da Silva et al. 2011; Koné et al. 2013; Lissemore
et al. 2006; Metcalfe et al. 2003; Peng et al. 2008), indicating effects of CBZ have been observed at environmentally relevant concentrations.

1.3 Zebrafish as a Model Organism

Zebrafish (*Danio rerio*) is a strong model organism for the study of endocrine disruption in fish and has been validated for use in several of the Organization for Economic Co-operation and Development’s (OECD) screening assays for endocrine disruption (http://www.oecd.org/env/ehs/testing/). Compared to other fish species there is a large knowledge base on the morphological, biochemical, and physiological characteristics at all stages from early development to adults of both sexes, providing a strong understanding of normal function for comparison to treated fish (Hill et al. 2005). Albeit, there are still some knowledge gaps that need to be filled before this organism can be fully optimized for endocrine disruption studies (Segner 2009). Their small size allows for easy handling and culturing in a laboratory setting, making them a viable option from a technical standpoint (Hill et al. 2005, Segner 2009). They have a high fecundity, with females producing between 50 to 300 eggs once a week, rapid development and a short generation time, reaching sexual maturity within 3 to 4 months, enabling reproductive, developmental (e.g. sexual differentiation) and transgenerational effects to be studied with relative ease (Hill et al. 2005; Segner 2009). In addition, the zebrafish genome is fully sequenced providing valuable information to study the changes in expression of genes important for steroids and reproduction. Furthermore, as molecular properties of the
zebrafish endocrine system and hormone signaling pathways are sufficiently similar to other vertebrates, zebrafish are appropriate for use in mechanistic research (Segner 2009).

1.4 The Zebrafish Testis

The structure of the mature testis is similar to that of mammals, being comprised of interstitial and tubular compartments (Grier 1981). The interstitial cells consist of the peritubular boundary cells and leydig cells. The tubules (Figure 1.1), sometimes showing an anastomosing pattern, are bound by a basement membrane, lined with a germinal epithelium and contain only sertoli and germ cells. The germ cells are arranged in spermatogenic cysts (Figure 1.1), with each cyst formed from a spermatogonium enveloped by sertoli cells. In zebrafish which have unrestricted testes, spermatogonia are randomly distributed along the length of the tubules (Grier et al. 1980; Grier 1981; Leal et al. 2009).

Within a cyst, synchronous development of the germ cells occurs as they progress through spermatogenesis. Spermatogonia will proliferate through mitosis (Figure 1.2A), and transform into primary spermatocytes with the onset of the first meiotic division (Figure 1.2B). The resulting secondary spermatocytes are short lived as they quickly enter meiosis II, producing spermatids. Upon completion of spermiogenesis, the mature sperm (spermatozoa) are voided into the tubular lumen (Figure 1.2C–G). As zebrafish have continuous reproduction, differing cysts can develop asynchronously and germ cells of
any stage can be found in the mature testis (Figure 1.2G; Grier et al. 1980; Grier 1981; Leal et al. 2009).

1.5 The Zebrafish Ovary

Zebras have a cystovarian, bilobed ovary. The ovarian wall is covered by a thin epithelium, over a connective tissue compartment, that projects ovigerous lamellae into the ovarian cavity (Figure 1.3). As zebras are asynchronous spawners, the ovigerous lamellae will contain oocytes in different growth stages, as well as atretic oocytes (Figure 1.4C) and post ovulatory follicles (Selman et al. 1993). In brief, oocyte growth can be divided into six main stages: oogenesis, primary oocyte growth (Figure 1.4A,B), cortical alveolus stage (Figure 1.4C–E), vitellogenesis (Figure 1.4F), maturation and ovulation (for detailed description in zebrafish see Selman et al. 1993 and for a review of teleosts see Tyler and Sumpter 1996). Oogonia will proliferate through mitosis and with the onset of meiosis I transform into a primary oocyte, where they begin the primary growth stage. The first part of the primary growth stage (IA) is marked by the presence of oocytes within nests, a large nucleus/nuclei to cytoplasm (ooplasm) ratio, and a threadlike appearance of chromosomes. In stage IB of primary growth meiosis I arrests in the diplotene stage of prophase I, the characteristic perinucleolar oocyte is observed as the nucleoli proliferate, decrease in size and move to the periphery of the nucleus; and the definitive follicle, which houses each oocyte individually, is formed (Figure 1.4A,B). The cortical alveolus stage is marked by the presence of cortical alveoli, membrane-limited vesicles that contain a
polysialoglycoprotein, which proliferate throughout this stage until they occupy the majority of the ooplasm (Figure 1.4C–E). The vitelline envelope also forms during this stage. Vitellogenesis follows with the sequestration of vitellogenin and production of yolk proteins that are sequestered in membrane-limited yolk bodies (Figure 1.4F). As vitellogenesis progresses, the cortical alveoli are displaced towards the periphery of the ooplasm. During maturation, meiosis I is reinitiated, the germinal vesicle breaks down, the first meiotic division occurs and meiosis II initiates and arrests in metaphase II. Lastly, ovulation occurs, and if fertilized, meiosis II will reinitiate and the second meiotic division will occur (Selman et al. 1993; Tyler and Sumpter 1996). At the time of fertilization, the cortical alveoli will release their content into the perivitelline space, acting to harden the vitelline envelope (chorion; Schuel 1978; Shibata et al. 2012) and prevent polyspermy (Schuel 1978; Ohta et al. 1990).

1.6 Sexual Differentiation in Zebrafish

Recent evidence suggests zebrafish have polygenic sex determination (also known as multigenic or multifactorial sex determination), whereby more than one genetic factor influences the sex, while environmental factors may influence the outcome in a secondary manner (Liew et al. 2012; Liew and Orbán 2014). It is not known how many and what genes are involved with regulating sex determination (Liew and Orbán 2014), but five different sex-associated regions have been identified between chromosomes 3, 4, 5 and 16 in three independent studies (Anderson et al. 2012; Bradley et al. 2011; Howe et al.
As no consistency of the regions was found between studies, it is possible there are differences across zebrafish strains with respect to the gene sets that play a role in sex determination (Liew and Orbán 2014). A suite of genes that have been implicated in sexual determination and differentiation in vertebrates have been identified in zebrafish, including \textit{amh, ar, cyp11b, cyp19a1a, dmrt1, ff1b, ff1d, nr0b1, sox9a}, and \textit{wt1a} (Hofsten and Olsson 2005; Orbán et al. 2009). Similarly multiple environmental factors have been found to influence sex determination in zebrafish including density (high density favouring increases in males; Liew et al. 2012), growth rate (with fast growth, as controlled with food, favouring female-biased sex ratios; Lawrence et al. 2008), oxygen levels (with hypoxia causing male-biased sex ratios; Shang et al. 2006) and temperature (higher temperatures leading to increases in males; Abozaid et al. 2011, 2012; Uchida et al. 2004; Villamizar et al. 2012).

In terms of differentiation, zebrafish are classified as juvenile hermaphrodites, first developing a juvenile ovary before it differentiates into a maturing ovary or testis (Maack and Segner 2003; Takahashi 1977; Uchida et al. 2002; Wang et al. 2007). The exact timing of sexual differentiation varies (Table 1.1), with the juvenile ovary phase starting and ending between 16 to 28 dpf and 23 to 42 dpf, respectively. The period of differentiation starts between 23 to 35 dpf and will finish as early as 43 and 48 dpf in males and females respectively (Maack and Segner 2003; Takahashi 1977; Uchida et al. 2002; Wang et al. 2007). Initially, the indifferent gonads consist of primordial germ cells (Figure 1.5A,B; see Yoshizaki et al. 2002 for a review in fish or Koç and Yuce 2012 for zebrafish specifics) and
their development (Figure 1.5C,D) into perinucleolar oocytes marks the subsequent juvenile ovary phase (Figure 1.5E–G). The onset of differentiation is characterized by apoptosis of the perinucleolar oocytes in presumptive males (Figure 1.6), while in presumptive females the perinucleolar oocytes will continue to grow and progress through the subsequent growth stages (Figure 1.4; Maack and Segner 2003; Takahashi 1977; Uchida et al. 2002). As differentiation progresses in presumptive males, alongside the degeneration of oocytes, an increase of stromal cells in the gonadal matrix can be observed (Figure 1.6) as well as early staged spermatogonial cysts. As the cysts progress through spermatogenesis (Figure 1.2) the stromal cells become peripherally located (Maack and Segner 2003; Takahashi 1977).

The exact mechanisms by which sexual differentiation initiates is unknown, however several signaling molecules and pathways have been found to be involved including tumour protein 53 (tp53) mediated apoptosis (Rodríguez-Marí et al. 2010), prostaglandins and prostaglandin synthase (Pradhan and Olsson 2014) and the “pro-female” signaling pathways NF-κB (Pradhan et al. 2012) and Wnt/β-catenin (Sreenivasan et al. 2014). Furthermore, it has been found that the germline is required for ovary fate determination (Slanchev et al. 2005) and a certain threshold number of germ cells must be present in order to enable female fate specification (Siegfried and Nüsslein-Volhard 2008).
1.7 Objectives

1.7.1 Timing of Sexual Differentiation in Untreated Fish

As studies have shown that the timeline for sexual differentiation can vary greatly in zebrafish depending on the strain and culture conditions of the laboratory (Maack and Segner 2003; Takahashi 1977; Uchida et al. 2002; Wang et al. 2007) the first objective was to determine the timeline for sexual differentiation within our own facilities. This will aid in determining appropriate time points for sampling in future studies as the timing of the morphological stages within sexual differentiation will be known. It was hypothesized that the selected time points of 15, 25, 35, 45, 60 and 75 dpf would allow for a characterization of the sexual differentiation timeline up to the development of distinguished males (i.e. testes) and females (i.e. maturing ovaries), but that full sexual maturity of the females would not be captured.

1.7.2 Sexual Differentiation in F1 Offspring of Exposed Parents

Previously in our lab, adult wildtype zebrafish were exposed to 0 (control, CTL) or 10 µg L^{-1} of CBZ or GEM for 6 weeks. Adults were then crossed for breeding to produce 7 distinct lineages: CTLM/CTLF, CBZM/CBZF, CTLM/CBZF, CBZM/CTLF, GEMM/GEMF, CTLM/GEMF, GEMM/CTLF (M = male, F = female). Male-biased sex ratios were noticed in the F1 offspring of most paternally exposed lineages at sexual maturity (CBZM/CBZF, CBZM/CTLF, GEMM/CTLF; Fraz and Wilson, unpublished data) suggesting the process of sexual differentiation was impacted from paternal exposure to these compounds. For
each lineage, offspring were sampled at 45 and 60 dpf to examine sexual differentiation. The second objective was to determine how sexual differentiation was impacted in the F_{1} offspring of the different lineages. It was hypothesized that more offspring with paternal exposure to CBZ or GEM would be at a male morphological stage of the sexual differentiation pathway by 60 dpf.
The first appearance of the morphological stages of sexual differentiation in zebrafish in days post fertilization (dpf) from the existing studies. The germ cells stage numbers indicate the first observation of germ cell(s) beyond primordial germ cells. The juvenile ovary stage numbers indicate the first observation of perinucleolar oocyte(s). Where morphological stages were not observed (N/O) the last time point of the study is indicated in brackets. Where the morphological stage would be present but the time point of the first appearance is unknown not applicable (N/A) is recorded.

<table>
<thead>
<tr>
<th>Morphological Stage</th>
<th>Takahashi 1977(^1)</th>
<th>Uchida et al. 2002(^1)</th>
<th>Maack and Segner 2003</th>
<th>Wang et al. 2007</th>
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<tbody>
<tr>
<td>Germ cells</td>
<td>13</td>
<td>13</td>
<td>28</td>
<td>14</td>
</tr>
<tr>
<td>Juvenile ovary</td>
<td>17</td>
<td>22</td>
<td>28</td>
<td>16</td>
</tr>
<tr>
<td>Transitioning</td>
<td>23</td>
<td>24</td>
<td>35</td>
<td>28</td>
</tr>
<tr>
<td>Perinucleolar oocytes maturing</td>
<td>23</td>
<td>N/A</td>
<td>35</td>
<td>28</td>
</tr>
<tr>
<td>Cortical alveolar growth oocytes</td>
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<td>53</td>
<td>N/O (77)</td>
<td>35</td>
</tr>
<tr>
<td>Vitellogenic oocytes</td>
<td>48</td>
<td>N/O (73)</td>
<td>N/O (77)</td>
<td>N/O (35)</td>
</tr>
<tr>
<td>Vitellogenic oocytes in all females</td>
<td>63</td>
<td>N/O (73)</td>
<td>N/O (77)</td>
<td>N/O (35)</td>
</tr>
<tr>
<td>Spermatogonia</td>
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<td>49</td>
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<tr>
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<td>49</td>
<td>35</td>
</tr>
<tr>
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<td>N/A</td>
<td>56</td>
<td>N/O (35)</td>
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<tr>
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<td>43</td>
<td>70</td>
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<tr>
<td>Spermatozoa in all males</td>
<td>63</td>
<td>43</td>
<td>N/O (77)</td>
<td>N/O (35)</td>
</tr>
</tbody>
</table>

\(^{1}\) Study time points were converted from days post hatch to dpf by assuming a hatch time of 3 dpf (Kimmel et al. 1995).
Figure 1.1. The structure of the testis in a sexually mature zebrafish. (A) Testis with (B) a tubule (larger circle) and spermatogenic cysts (smaller circles) at the germinal epithelium highlighted. Spermatozoa fill the lumen. Hematoxylin and eosin staining; 40x.
Figure 1.2. The succession of spermatogenesis in developing zebrafish testes. (A) Early testis with spermatogonia (sg, 35 days post fertilization, dpf). (B) Early testis with spermatogonia and spermatocytes (sc, 45 dpf). (C) Small lumens with spermatozoa (sz) just starting to form (45 dpf). (D) Growing lumens but still dominated by spermatocyte cysts (45 dpf). (E) Testis highlighting a spermatid (sd) cyst bordering a lumen and (F) an overview of the same testis (60 dpf). (G) A fully mature testis dominated by spermatozoa (75 dpf). Hematoxylin and eosin staining; 50 µm = 40x, 100 µm = 20x, 200 µm = 10x. li = liver, pan = pancreas, int = intestine.
Figure 1.3. The structure of the ovary in a juvenile zebrafish (45 days post fertilization). (A) Ovary with (B) ovigerous lamellae (black outline around oocytes) projecting into the ovarian cavity (oc) from the epithelium over the ovarian wall (ow). The ovigerous lamellae contain only perinucleolar (primary growth stage) oocytes. Hematoxylin and eosin staining; 10x. li = liver, int = intestine.
**Figure 1.4.** Oocyte growth in developing zebrafish ovaries. (A) Maturing perinucleolar oocytes (po) where early forming perinucleolar oocytes (*) and germ cell clusters (circle) can be observed in-between (35 days post fertilization, dpf). (B) An overview of part of an ovary with maturing perinucleolar oocytes (po, 60 dpf). (C) An atretic oocyte (arrow) and an early cortical alveolar growth oocyte (ca) with small cortical alveoli at the periphery (75 dpf). Atresia has not been confirmed with TUNEL positive staining but is based on appearance only. (D) A cortical alveolar growth oocyte (centre) with large and numerous cortical alveoli progressed towards the nucleus (75 dpf). (E) An overview of part of an ovary filled with cortical alveolar growth oocytes in addition to perinucleolar oocytes (75 dpf). (F) An early vitellogenic oocyte (centre) with eosinophilic lipid bodies accumulating between the nucleus and cortical alveoli in a fish at sexual maturity. n = nucleus, o = ooplasm. Hematoxylin and eosin staining; 50 µm = 40x, 100 µm = 20x, 200 µm = 10x.
Figure 1.5. The undifferentiated stages of gonad development in zebrafish. (A) and (B) Primordial germ cell clusters as seen from different sections in a 15 dpf fish. (C) A thick and (D) thin strip of germ cells at various stages in fish at 25 dpf. (E) Perinucleolar oocytes (po) just beginning to form in what is now a juvenile ovary at 25 dpf and (F) an overview of the same ovary. (G) A juvenile ovary at 35 dpf with more developed but still sparse perinucleolar oocytes. It is uncertain whether they will continue to grow or degenerate. Hematoxylin and eosin staining; 20 µm = 100x, 50 µm = 40x, 100 µm = 20x. li = liver, int = intestine.
Figure 1.6. Gonads transitioning from the juvenile ovary to testes in zebrafish. (A) An ovary transitioning at 35 days post fertilization (dpf) marked by limited perinucleolar oocytes (po) and an increase of stromal cells in the central region; (B) highlights the isolation of the perinucleolar oocytes as a result. (C) and (D) Fish undergoing a transition at 25 dpf, with perinucleolar oocytes demonstrating morphological changes of degeneration including an increased basophilia of the ooplasm and off-coloured patches in the ooplasm. An increase in stromal cells can be seen, as well as the presence of germ cells (gc). Hematoxylin and eosin staining; 20 μm = 100x, 50 μm = 40x, 100 μm = 20x. li = liver, int = intestine.
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CHAPTER 2
THE EFFECTS OF PARENTAL CARBAMAZEPINE AND GEMFIBROZIL EXPOSURE ON SEXUAL DIFFERENTIATION IN ZEBRAFISH (*Danio rerio*)

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ABSTRACT

Parental exposure to 10 µg L\(^{-1}\) of the pharmaceuticals carbamazepine or gemfibrozil led to male-biased sex ratios in the sexually mature F\(_1\) offspring of zebrafish (*Danio rerio*), suggesting that sexual differentiation may be impacted by parental exposure. Since the timing of sexual differentiation has been shown to be dependent on culture conditions, we first established this for our facility. Untreated fish were bred and offspring were reared and sampled at six time points between 15 to 75 days post fertilization (dpf). Whole fish were prepared for histological analysis, serial sectioned, stained with hematoxylin and eosin and reviewed under light microscopy. Gonads were classified into morphological stages based on gamete stages. In untreated fish, sexual differentiation began as early as 25 dpf and by 60 dpf all fish were found to be on a male or female differentiation pathway. We then examined sexual differentiation in the F\(_1\) offspring from carbamazepine or gemfibrozil exposed parents at 45 and 60 dpf. Paternal, but not maternal, exposure to carbamazepine resulted in a significantly faster sexual differentiation (more fish with spermatozoa at 45 dpf) and led to a male-biased sex ratio; these effects were not observed when both parents were exposed. Combined paternal and maternal exposure to gemfibrozil resulted in a significantly faster sexual differentiation (more fish with spermatozoa at 45 dpf) and paternal, but not maternal, exposure to gemfibrozil led to male-biased sex ratios. Interestingly, sex ratios observed in the juveniles did not always reflect those found at sexual maturity, suggesting a sex reversal, including a male to female transition, occurred in some fish past the juvenile sexual differentiation period. This study demonstrates the ability of parental exposure to pharmaceuticals to disrupt sexual differentiation in the F\(_1\) offspring of exposed parents.
2.1 INTRODUCTION

Endocrine-disrupting compounds are “natural or man-made substances that disrupt function, levels, and distribution of endogenous hormones of exposed organisms by either mimicking or antagonizing the actions of hormones, or by modulating hormone synthesis or metabolism” (Segner 2009). Traditionally, pharmaceuticals have not been thought of as endocrine disruptors with the exception of steroidogenic compounds such as 17α-ethinylestradiol (EE2; Andersen et al. 2003; Fenske et al. 2005; Jobling et al. 1996; Kidd et al. 2007; Länge et al. 2001; Örn et al. 2003, 2006; Purdom et al. 1994; Scholz and Gutzeit 2000; van Aerle et al. 2002; Xu et al. 2008), 17α-methyltestosterone (Fenske and Segner 2004; Örn et al. 2003), and the anti-estrogen fadrozole (Ankley et al. 2002; Fenske and Segner 2004; Kwon et al. 2000; Panter et al. 2004). However, several pharmaceuticals which do not have a mechanism of action that directly disrupts steroid production or interacts with steroid receptors have been found to alter sex steroid levels and impair reproduction in fish, including fluoxetine (Foran et al. 2004; Lister et al. 2009; Mennigen et al. 2010); propranolol (Huggett et al. 2002); clofibric acid (active metabolite of clofibrate; Runnalls et al. 2007); carbamazepine (Galus et al. 2013a, 2014) and gemfibrozil (Galus et al. 2013a, 2014; Mimeault et al. 2005).

Pharmaceuticals can be found in surface waters in the ng L\(^{-1}\) to µg L\(^{-1}\) range (Brun et al. 2006; Benotti and Brownawell 2007; da Silva et al. 2011; Kolpin et al. 2002; Koné et al. 2013; Lissemore et al. 2006; Metcalfe et al. 2003; Peng et al. 2008; Stumpf et al. 1999).
Carbamazepine (CBZ) has been detected in surface waters at concentrations as high as 0.7 µg L\(^{-1}\), with reported median concentrations falling in the range of 0.001–0.185 µg L\(^{-1}\) (Benotti and Brownawell 2007; da Silva et al. 2011; Koné et al. 2013; Lissemore et al. 2006; Metcalfe 2003). CBZ is primarily used in the treatment of epilepsy and bipolar disorder. As an anticonvulsant, the main targets for CBZ are voltage-gated sodium channels (Ambrósio et al. 2002) while as an antiepileptic it is believed to target the brain arachidonic acid cascade (Rao et al. 2008). There is still much to be learned about its mechanism of action(s), and it’s likely there are multiple mechanisms of action that are responsible for its therapeutic effects (Ambrósio et al. 2002; Rao et al. 2008). In adult zebrafish (*Danio rerio*), exposure to 0.5 µg L\(^{-1}\) of CBZ for 6-weeks decreased plasma 11-ketotestosterone levels in males and females by 9.5-fold and 2.2-fold, respectively (Galus et al. 2013a). In the common carp (*Cyprino carpio L*.), CBZ significantly impacted spermatozoa at doses of 2.0 and 20 mg L\(^{-1}\) after a 2 h incubation, causing decreased motility and velocity, and induced oxidative stress (Li et al. 2010).

Gemfibrozil (GEM) has been detected in surface waters at concentrations as high as 0.79 µg L\(^{-1}\), with reported median concentrations falling in the range of 0.012–0.066 µg L\(^{-1}\) (Brun et al. 2006; da Silva et al. 2011; Kolpin et al. 2002; Koné et al. 2013; Metcalfe et al. 2003; Peng et al. 2008; Stumpf et al. 1999). GEM is a fibrate lipid regulator, used for treating hypertriglyceridemia and combined hyperlipidemia (Roy and Pahan 2009). GEM activates the peroxisome proliferator-activated receptor α (PPARα; Forman et al. 1997), which acts to regulate lipid and lipoprotein metabolism, and more recently has been
shown to have anti-inflammatory activity (Kota et al. 2005; Chinetti et al. 2010). GEM was shown to reduce plasma testosterone by 50% in male goldfish (*Carassius auratus*) following exposure to 1.5 µg L\(^{-1}\) of gemfibrozil (GEM) for 14 days (Mimeault et al. 2005). We have documented reduced 11-ketotestosterone in male zebrafish with exposure to 10 µg L\(^{-1}\) for 6 weeks (Fraz and Wilson, unpublished data).

Zebrasfish (*Danio rerio*) is a strong model organism for the study of endocrine disruption in fish and has been validated for use in several of the Organization for Economic Co-operation and Development’s (OECD) screening assays for endocrine disruption ([http://www.oecd.org/env/ehs/testing/](http://www.oecd.org/env/ehs/testing/)). Recent evidence suggests zebrafish have polygenic sex determination (also known as multigenic or multifactorial sex determination), while environmental factors may influence the outcome in a secondary manor (Liew et al. 2012; Liew and Orbán 2014). Currently it is not known what genes or how many regulate sex determination (Liew and Orbán 2014). Density (Liew et al. 2012), growth rate (Lawrence et al. 2008), oxygen levels (Shang et al. 2006) and temperature (Abozaid et al. 2011, 2012; Uchida et al. 2004; Villamizar et al. 2012) have been implicated as environmental factors which can influence sex determination. The potential for non-steroidogenic pharmaceuticals to influence sex determination in zebrafish is not clear however, chemical exposures have altered sex ratios (Baker et al. 2014; Nakari and Erkomaa 2003).
In terms of sexual differentiation, zebrafish are classified as juvenile hermaphrodites, first developing a juvenile ovary before it differentiates into a maturing ovary or testis through apoptosis (Maack and Segner 2003; Takahashi 1977; Uchida et al. 2002; Wang et al. 2007). The exact timing of sexual differentiation varies, with the juvenile ovary stage, marked by the presence of perinucleolar oocytes, starting and ending between 16 to 28 days post fertilization (dpf) and 23 to 42 dpf, respectively (Maack and Segner 2003; Takahashi 1977; Uchida et al. 2002; Wang et al. 2007). Throughout this time, the gonad is considered to be undifferentiated. The period of differentiation starts between 23 to 35 dpf and ends as early as 43 and 48 dpf in males and females, respectively (Maack and Segner 2003; Takahashi 1977; Uchida et al. 2002; Wang et al. 2007). In males, differentiation is initiated with a gonadal transition involving the degeneration of the oocytes and an increase in stromal tissue (Maack and Segner 2003; Takahashi 1977; Uchida et al. 2002). The presence of germ cells in cyst-like clusters indicates the start of the early testes (Maack and Segner 2003). Spermatogonia will develop and progress through spermatogenesis, from spermatocytes to spermatids to spermatozoa (see Grier 1981 or Leal et al. 2009); subsequently displacing the stromal cells to the periphery (Maack and Segner 2003; Takahashi 1977). In females, the differentiation pathway can first be identified with the maturing of perinucleolar oocytes which will progress to cortical alveolar growth and vitellogenesis before reaching maturation (see Selman et al. 1993 for specifics on oocyte growth in zebrafish or Tyler and Sumpter 1996 for a review in teleosts).
Over the course of a 6-week exposure, direct exposure of adult zebrafish to CBZ or GEM reduced fecundity at 0.5 and 10 µg L$^{-1}$, induced histopathological changes to the ovary starting at 0.5 µg L$^{-1}$, and reduced the number of mature follicles at 10 µg L$^{-1}$ (Galus et al. 2013a). In the F$_1$ offspring of zebrafish whose parents were exposed to 10 µg L$^{-1}$ of CBZ or GEM for 4 weeks, reduced fecundity as well as altered courtship, sperm velocity and sperm morphology have been observed in adulthood (Galus et al. 2014). In this study, adult wildtype zebrafish were exposed to 0 (control, CTL) or 10 µg L$^{-1}$ of CBZ or GEM for 6 weeks. Adults were then crossed for breeding to produce 7 distinct lineages: CTLM/CTLF, CBZM/CTLF, CTLM/CBZF, CBZM/CTLF, GEMM/GEMF, CTLM/GEMF, GEMM/CTLF (M = male, F = female). Male-biased sex ratios were noticed in the F$_1$ offspring at sexual maturity for lineages with paternal exposure (CBZM/CBZF, CBZM/CTLF, GEMM/CTLF; Fraz and Wilson, unpublished data), suggesting that sexual differentiation of the gonads could be influenced by parental exposure to these compounds. The purpose of this study was two-fold. First, as the timing of sexual differentiation can vary in zebrafish, particularly with culture conditions, the first objective was to establish the timing of sexual differentiation within our zebrafish culture. Second, we examined sexual differentiation in the juvenile F$_1$ offspring generated by crossing control, GEM, or CBZ exposed zebrafish; juveniles from the 7 lineages described above. Of the latter we addressed two issues. First, we determined if there was a difference in the timing of sexual differentiation between control and treatment lineages at 45 and 60 dpf. Second, we examined whether there was a difference in the sex ratio between control and treatment lineages at 60 dpf. It was
hypothesized that by 60 dpf more offspring with paternal exposure to CBZ or GEM would be at a male morphological stage of the sexual differentiation pathway.

2.2 MATERIALS & METHODS

2.2.1 Adult Zebrafish Culture

Adult wildtype zebrafish were obtained from AQUALity Tropical Wholesale, Inc. (Mississauga, ON, Canada). Fish were held in 10 L tanks at 50 fish tank\(^{-1}\) in a 1:1 sex ratio on a multi-rack research system from Aquatic Habitats\(^\text{®}\). System water was composed of distilled water with 60 mg L\(^{-1}\) Instant Ocean Salt (Spectrum Brand, USA) and 120 mg L\(^{-1}\) sodium bicarbonate (Aquatic Eco System Inc., USA) with 10% renewal each day. Water parameters were maintained at 27–30\(^\circ\)C, pH 6.5–7.5, 80–100% dissolved oxygen, and 300–400 \(\mu\)S through constant monitoring (YSI 5200, YSI Inc., USA) zebrafish were fed twice a day with flakes (Nutrafin Max Tropical Fish Flakes, Hagen, Canada and Staple Flake Food, Big Al’s, Canada) and once a day with live Artemia (Artemia Cysts, INVE Aquaculture Nutrition, USA). The photoperiod was maintained at 14 h: 10 h light:dark.

2.2.2 \(F_1\) Offspring from Untreated Fish

Breeding traps (plastic container with mesh screen) were dropped into adult tanks of untreated fish prior to the onset of breeding at first light and embryos were collected 1.5 h post light over 3 successive mornings. Embryos were transferred to 2 L plastic containers at 150 embryos container\(^{-1}\) and held in an incubator maintained at 28.5\(^\circ\)C. Embryos were initially kept in 1X E3 medium (5 mM sodium chloride, 0.17 mM potassium...
chloride, 0.33 mM magnesium sulphate and 0.33 mM calcium chloride) and gradually transferred to system water between 12 and 16 dpf (i.e. 10%, 25%, 50%, 80%, and 100% system water on successive days). Water depth was initially maintained at 2.5 cm and increased to 6.5 cm by 1 cm per day between 12 and 16 dpf. Water change-outs occurred daily with 90% renewal. At 19 dpf fish were transferred to 3 L tanks at a density of 50 fish tank\(^1\).

Feeding commenced at 5 dpf with 4 feeds per day of a 50:50 mixture of finely crushed fish flakes and Grade 0 Hatch Fry Encapsulation (Argent Chemical Laboratories, USA) at a minimum 2 h interval. Between 9 to 15 dpf fish were fed twice daily with a 50:50 mixture of crushed flakes and Grade 0 Hatch Fry Encapsulation and twice daily with first instar Artemia. Between 15 to 30 dpf and 30 to 45 dpf the 50:50 mixture was switched to crushed flakes with Grade 1 and Grade 3 Hatch Fry Encapsulation, respectively. From 45 dpf onwards, the 50:50 mixture was replaced with 2 feeds per day of moderately coarse flakes. From 30 to 75 dpf feeds decreased to 3 per day with only 1 feed per day of adult Artemia.

At 15, 25, 35, 45, 60 and 75 dpf 30 fish were sampled randomly from 8 independent tanks as determined by a random number generator and 20 fish from each time point were prepared for histological analysis.
2.2.3 Exposure of Parental Fish to Carbamazepine and Gemfibrozil

Breeding output for each tank of the adult culture was monitored daily for 15 days. Breeding traps were dropped into tanks prior to the onset of breeding at first light and the number of eggs per tank were counted 1.5 h after first light. After the monitoring period 9 tanks with similar reproductive output were selected and randomly distributed to treatments.

Fish were housed in triplicate 12 L glass aquaria at a density of 3 fish L\(^{-1}\) and a 1:1 sex ratio. Water chemistry was maintained as described above. Tanks were recirculating and maintained at a temperature of 28–29°C with 90% water renewal every 3 days for the duration of the exposure. Temperature and pH were monitored daily, and weekly measurements of conductivity, dissolved oxygen (using portable YSI, YSI Inc., USA), nitrate, nitrite, general hardness, carbonate hardness and ammonia were conducted (using Nutrafin water quality test kit). Fish were fed and kept to the same light regime as the adult culture. Dosing solutions of carbamazepine (CBZ) and gemfibrozil (GEM; Sigma Aldrich, Canada) were prepared in DMSO and added to exposure tanks to obtain a minimal concentration of 10 µg L\(^{-1}\). The final concentration of DMSO in all the tanks was ≤0.004%. Fish were exposed for 42 days prior to the beginning of pairwise breeding.

2.2.4 F\(_1\) Offspring from Parentally Exposed Fish

The F\(_1\) offspring were produced from pairwise breeding of one male and one female. Crosses were completed within treatment, where both parents (male, M or
female, F) were exposed (CBZM/CBZF, GEMM/GEM; carbamazepine, CBZ or gemfibrozil, GEM) or unexposed (CTLM/CTLF; CTL, control). Additional, reciprocal crosses were completed where only a single parent was exposed (i.e. CTLM/CBZF, CTLM/GEMF, CBZM/CTLF and GEMM/CTLF). Breeding took place over 18 days with a total of 75 pairs bred for each pairwise and reciprocal cross. Males and females were not paired together twice.

For each breeding event a male and female were held overnight in a plastic pairwise breeding tank (700 mL) separated by a divider. Breeding tanks were filled with clean system water and maintained at 28.5°C. In the morning the divider was removed shortly after first light and the pair was provided an opportunity to breed. Following the breeding opportunity (1.5 hours after first light), fish were returned to exposure tanks, i.e. control or 10 µg L\(^{-1}\) of CBZ or GEM.

The rearing of the F\(_1\) offspring from the parental exposure followed the rearing protocol described above with the following exceptions. Embryos were transferred to 2 L plastic containers at a density of 250 embryos container\(^{-1}\) with 1X E3 medium approximately 2 cm deep. Fish were transferred to 3 L tanks at 30 dpf. Between 21 to 30 dpf and 30 to 60 dpf the 50:50 mixture of finely crushed flakes and Grade 0 Hatch Fry Encapsulation was switched to Grade 1 and Grade 3 Hatch Fry Encapsulation, respectively. From 35 dpf feeds decreased to 3 feeds per day with 2 feeds per day of the 50:50 mixture.
and 1 feed per day of adult Artemia. Starting at 60 dpf the 50:50 mixture was replaced with moderately coarse flakes.

At 45 and 60 dpf, 48 fish were randomly sampled from each lineage (CTLM/CTLF, CBZM/CBZF, CTLM/CBZF, CBZM/CTLF, GEMM/GEMF, CTLM/GEMF, GEMM/CTLF) and 32 fish were prepared for histological analysis.

2.2.5 Histology

Fish were anaesthetized with MS-222 and fixed in 10% neutral buffered formalin for a minimum of 48 h, followed by a 50% ethanol rinse for 30 min before transfer to 70% ethanol for storage. Measurements of total length were recorded for the offspring of untreated parents from 15 to 35 dpf by microscope (AxioCam HSm and Zen Pro 2012) and from 45 to 75 dpf by hand. Fish were embedded as four per cassette in paraffin wax at McMaster University’s Core Histology Suite. Cassettes were sectioned sagittally at 5 µm by microtome with approximately 8 sections per cassette to ensure that gonads were captured from all fish and proper scoring of the sexual differentiation stage could be conducted (Maack and Segner 2003). All sections were stained with hematoxylin and eosin-Y (Richard-Allan Scientific, USA), mounted in permount and reviewed by light microscopy to determine the sexual differentiation stage of each fish based on gonad morphology (see Table 2.1 for morphological stage). Selman et al. (1993) was used to identify and determine the growth stages of the oocytes while van der Ven and Wester (http://www.rivm.nl/fishtoxpat/) was used to help guide the staging of male germ cells. If
there was uncertainty as to the morphological stage (e.g. borderline between two categories) the lower stage was recorded. Fish with sections where the gonad was not captured or with insufficient evidence to determine the morphological stage (i.e. too small a piece of gonad) were eliminated from analyses. Slides were read blind (for age and lineage, sections from the same individual were known) by a single assessor (K. Hammill), while select slides were reviewed blind by a second assessor (J. Wilson) to confirm the morphological stages. Within offspring of each lineage between 0–5 granulomas and 0–4 patches of eosinophilic fluid, with no more than 1 per gonad, were observed. As no differences could be attributed to treatment effects and granulomas are known to occur spontaneously in ovaries (Rossteuscher et al. 2008) these observations were not considered further.

2.2.6 Statistical Analysis

A day-equivalent (weighted mean in days post fertilization) was calculated for each morphological stage from the untreated fish sampled from 15 to 75 dpf based on the occurrence of a given morphological stage at each time point (Table 2.2). The day-equivalent was calculated according to the equation:

$$\bar{X} = \frac{\sum_{i=1}^{N} x_i w_i}{N}$$

where $\bar{X}$ is the day-equivalent, $x_i$ is the time point (in dpf), $w_i$ is the occurrence of a given morphological stage at $x_i$, and $N$ is the total occurrence of a given morphological stage. A
sample calculation for the day-equivalent can be found in the Appendix. The day-equivalent was then substituted for the morphological stage of the gonad observed for each of the F_1 offspring, and a mean day-equivalent for each lineage was calculated to provide a quantitative score for analysis (Table 2.3). The mean day-equivalent was calculated according to the equation:

\[ \bar{X}_L = \frac{\sum_{i=1}^{N} \text{Day Equivalent}}{N} \]

where \( \bar{X}_L \) is the mean day-equivalent for a lineage and \( N \) is the total number of fish in the lineage. A sample calculation for the mean day-equivalent can be found in the Appendix. Normality was verified for 45 dpf data via bootstrap (with replacement, \( n = 1000 \)) sampling distributions and confidence intervals. Data from 60 dpf showed slight deviations from normal distribution but given the robustness of the ANOVA (Schmider et al. 2010) parametric testing was deemed appropriate. Equal variance was verified via examination of residual plots. To determine if there was a difference in the timing of sexual differentiation for the F_1 offspring a two-way ANOVA (male parent treatment vs. female parent treatment) was run on the data separately for each compound (CBZ or GEM) and time point (45 or 60 dpf). Where significant effects of the factors and/or interaction was found pairwise comparisons were conducted with t-tests with the Bonferroni correction applied. To determine whether or not the treatment of the male or female parent, or an interaction of the two, impacted sex ratio, a logistic regression was conducted on the data from 60 dpf separately for CBZ and GEM lineages. The one fish at
the perinucleolar oocytes juvenile stage was excluded from this analysis. All statistical analyses were performed in R 3.3.1 and a significance level of p < 0.05 was used.

2.3 RESULTS

2.3.1 Timing of Sexual Differentiation in Untreated Fish

The germ cell stage was only found at 15 dpf (3.9–7.4 mm total length, \( L_T \), \( n = 15 \)) and 25 dpf (4.2–12 mm \( L_T \), \( n = 16 \); Figure 2.1). At both 15 and 25 dpf, several fish were found to have primordial germ cell clusters while the majority had germ cells at various stages. The first juvenile ovaries and transitioning gonads were observed at 25 dpf. By 35 dpf (9.3–16 mm \( L_T \), \( n = 19 \)) the first distinctively male and female gonads were present. This was marked by the first early stage testes in males as well as maturing perinucleolar oocytes and cortical alveolar growth oocytes in females. The early stage testes were characterized by germ cells arranged in a cyst like manner and/or the presence of spermatogonia. At 45 dpf (10–20 mm \( L_T \), \( n = 20 \)) the first males with spermatozoa were observed while the early testes had predominately spermatogonia with some presumptive spermatocytes. By 60 dpf (13–21 mm \( L_T \), \( n = 20 \)) all fish were on a male or female differentiation pathway and no undifferentiated stages were found. By 75 dpf (12–22 mm \( L_T \), \( n = 20 \)) spermatozoa was present in all males and cortical alveolar growth oocytes were observed in 86% of females. No vitellogenic oocytes were observed at any time point. A few cortical alveolar oocytes appeared to be undergoing atresia in 2 fish at 60 dpf and 7 fish at 75 dpf.
2.3.2 Timing of Sexual Differentiation in F₁ Offspring of Exposed Parents

The day-equivalent was calculated for each morphological stage (Table 2.2) and the respective day-equivalent was substituted for each occurrence of a morphological stage in the F₁ offspring reared after parental exposure to carbamazepine (CBZ) or gemfibrozil (GEM). This allowed for the calculation of a mean day-equivalent (Table 2.3) for each lineage and a quantitative comparison in the timing of sexual differentiation. Table 2.3 provides a comparison of the timing of sexual differentiation between lineages and the calculated mean day-equivalent by lineage. Figures 2.2 and 2.4 describe the distribution of the morphological stages at 45 and 60 dpf, respectively.

At 45 dpf (n = 30) a mean day-equivalent of 45 (Table 2.3) was determined for the control F₁ offspring (CTLM/CTLF). Several offspring (3) were still at the perinucleolar oocytes (po) juvenile stage while all presumptive females were at the po maturing stage (Figure 2.2). The males were spread amongst the four categories of transitioning, early testis, spermatids and spermatozoa (Figure 2.2, 2.3), with about half of the males (7) at the early testis stage. By 60 dpf (n = 32) a mean day-equivalent of 59 (Table 2.3) was determined for the control F₁ offspring. All offspring were on a male or female differentiation pathway (Figure 2.4).

At 45 dpf both the treatment of the father (p = 0.001) and mother (p = 0.022) were found to be significant factors in the rate of sexual differentiation for the CBZ F₁ lineages, and an interaction was found (p = 0.021). Offspring with paternal (CBZM/CTLF, n = 30),
but not maternal, exposure underwent sexual differentiation at a significantly faster rate than offspring of unexposed parents (CTLM/CTLF, \( p < 0.001 \)). The mean day-equivalent for offspring from the CBZM/CTLF lineage was 54 dpf (Table 2.3), well ahead of their actual age at sampling. This was noticeable in male offspring of which the majority were at the spermatozoa stage (Figure 2.2, 2.3). However, when both parents were exposed (CBZM/CBZF, \( n = 32 \), mean day-equivalent 46), the rate of sexual differentiation in offspring was not different from offspring of unexposed parents (\( p = 1.00 \)) and was significantly slower than the offspring with paternal but not maternal exposure (\( p = 0.008 \)). Maternal exposure alone (CTLM/CBZF; \( n = 32 \), mean day-equivalent 45) did not impact the rate of sexual differentiation. By 60 dpf no significant differences in the rate of sexual differentiation were found between any of the CBZ and control lineages (Table 2.3).

Both the treatment of the father (\( p = 0.016 \)) and the mother (\( p < 0.001 \)) were found to be significant factors in the rate of sexual differentiation for the GEM F1 lineages. When both parents were exposed (GEMM/GEMF, \( n = 32 \)), the F1 offspring had a significantly faster rate of sexual differentiation (mean day-equivalent 55) than offspring with unexposed parents (CTLM/CTLF; \( p < 0.001 \)) at 45 dpf (Table 2.3). This was characterized by a greater presence of male offspring at the spermatozoa stage (Figure 2.2, 2.3). Neither the paternal only (GEMM/CTLF; \( n = 31 \), mean day-equivalent 48) or maternal only (CTLM/GEMF; \( n = 32 \), mean day-equivalent 50) exposed offspring differed in their rate of sexual differentiation as compared to that of the control. By 60 dpf no significant
differences in the rate of sexual differentiation were found between any of the GEM and control lineages (Table 2.3).

2.3.3 Sex Ratio in F1 Offspring of Exposed Parents

The offspring of unexposed parents (CTLM/CTLF) displayed a relatively even sex ratio of 47% males and 53% females (Figure 2.5). Paternal exposure to CBZ was found to lead to male-biased sex ratios (p = 0.015) in the offspring although this was dependent upon the maternal exposure condition (interaction p = 0.005). In the offspring with paternal exposure only (CBZM/CTLF) males were dominant with 77% males and 23% females, while offspring where both parents were exposed (CBZM/CBZF) were similar to controls with 48% males and 52% females. Paternal exposure to GEM (p = 0.029), regardless of the maternal exposure, was found to lead to male-biased sex ratios. Offspring were 74% males and 26% females with paternal exposure alone, while 100% males were observed in offspring with combined maternal and paternal exposure.

2.4 DISCUSSION

2.4.1 Timing of Sexual Differentiation in Untreated Fish

Zebrafish is a major model organism for endocrine disruption studies in fish, making it necessary to have an understanding of the process of sexual differentiation and the accompanying morphology of their gonads. Several past studies have characterized the differentiation of the juvenile ovary (Maack and Segner 2003; Takahashi 1977) but the timing has been found to vary greatly between studies (Maack and Segner 2003;
Takahashi 1977; Uchida et al. 2002; Wang et al. 2007). This is thought to be dependent on culture conditions of the laboratory and strain (Segner et al. 2003). Although, it should be noted that a greater variability can be observed between individuals within a strain than between two strains (Maack et al. 2003). Due to the variation, it is necessary to determine the timing of sexual differentiation under the culture conditions of each facility.

Overall the initial appearance of each morphological stage in gonads of fish from our facility fell within the range of timing for what has been previously documented (Maack and Segner 2003; Takahashi 1977; Uchida et al. 2002; Wang et al. 2007). Germ cells, beyond that of primordial germ cells, have been documented as early as 13 dpf (Takahashi 1977; Uchida et al. 2002), while in our study this was observed at 15 dpf, the earliest time point examined. The first juvenile ovaries and transitioning gonads were observed at 25 dpf, with the latter marking the onset of differentiation, which is known to start between 23 to 35 dpf (Maack and Segner 2003; Takahashi 1977; Uchida et al. 2002; Wang et al. 2007). All fish were found to have a maturing ovary or testis by 60 dpf. The odd transitioning gonad has still been observed as late as 77 dpf (Maack and Segner 2003) but in general distinguished ovaries and testes are present by 60 dpf (Maack and Segner 2003; Takahashi 1977; Uchida et al. 2002; Wang et al. 2007).

In our facility, spermatozoa were first noted at 45 dpf and were present in all males by 75 dpf. The first appearance of spermatozoa has been documented as early as 43 dpf (Takahashi et al. 1977; Uchida et al. 2002) and as late as 70 dpf (Maack and Segner 2003).
Testes have been completely formed as early as 43 dpf (Uchida et al. 2002). For females, vitellogenic oocytes were not observed at any time point and cortical alveolar growth was the most advanced stage present. Although Takahashi (1977) observed vitellogenic oocytes in females as early as 48 dpf, Maack and Segner (2003) found cortical alveolar growth and vitellogenic oocytes absent even by 77 dpf. Wang et al. (2007) observed cortical alveolar growth oocytes by 35 dpf, the time point for which they were first noted in this study. Altogether, these observations validated the time points of 45 and 60 dpf to determine differences in the timing of sexual differentiation, as well as the latter to determine differences in sex ratios.

2.4.2 Sexual Differentiation in F₁ Offspring of Exposed Parents

Sexual differentiation occurs through molecular, genetic and physiological mechanisms given the genotype and environment of an organism (Piferrer and Guiguen 2008) and encompasses gonadogenesis and gametogenesis. In fish, sexual differentiation occurs post hatch (Maack and Segner 2003; Nakamura 1984; Nakamura et al. 1998; van Aerle et al. 2004) and is thought to be a heightened time of sensitivity to endocrine-disrupting compounds (Andersen et al. 2003; van Aerle et al. 2002). Direct exposure to the pharmaceutical EE2 during the period of sexual differentiation has been shown to negatively impact this process in fish including inhibiting testicular growth (in rainbow trout, Oncorhynchus mykiss, Jobling et al. 1996); altering the proportion of germ cell stages (in rainbow trout, Jobling et al. 1996; in zebrafish, Xu et al. 2008), reducing the
number of spermatozoa in testes and disrupting sperm duct development (in fathead minnow, *Pimephales promelas*, van Aerle et al. 2002; in zebrafish, Xu et al. 2008). However, few studies have examined the impact of parental exposure to pharmaceuticals on the F1 offspring (Parrot and Bennie 2009; Galus et al. 2014), leaving it unknown how sexual differentiation can be impacted with parental pharmaceutical exposure.

In the present study, a subset of a multi-generational study, pairwise and reciprocal crosses of parents exposed to 10 ug L\(^{-1}\) of carbamazepine (CBZ) or gemfibrozil (GEM) was found to cause male-biased sex ratios in F1 offspring at sexual maturity with paternal CBZ exposure (CBZM/CBZF, CBZM/CTLF lineages) and paternal GEM, but not maternal (GEMM/CTLF lineage) exposure. As such we had the objective of examining sexual differentiation in the juvenile F1 offspring.

2.4.3 Timing of Sexual Differentiation in F1 Offspring of Exposed Parents

In our study, the first question examined was whether or not there was a difference in the timing of sexual differentiation in the F1 offspring, i.e. were gonads developing faster, slower or at the same rate as that in offspring of unexposed parents. Rather than just examining qualitative differences in the morphological stages of the developing gonads, we wanted to quantitatively assess how timing was impacted. In order to do this, we needed a method to convert the morphological stages (categorical observations) into a quantitative value. A day-equivalent was used to numerically represent each morphological stage, enabling a quantitative analysis of the timing of
sexual differentiation. To the authors’ knowledge this is the first time this style of analysis has been applied to quantify differences in the timing of sexual differentiation. An ANOVA was conducted which offers the advantage of generating more statistical power as compared to categorical tests, and enabling the testing of interactions, i.e. between paternal and maternal effects.

Paternal exposure to CBZ was found to cause a faster sexual differentiation of offspring, but only when there was no maternal exposure, indicating a potential protective effect of maternal exposure (Table 2.3, Figure 2.2). Maternal effects of chemical stressors have rarely been studied in non-mammalian vertebrates and the mechanism of how maternal pharmaceutical exposure could be exerting a beneficial effect is unknown. This was not observed for GEM, where combined paternal and maternal exposure led to a faster sexual differentiation in the offspring.

Direct exposure to EE2 during differentiation has been found to suppress the process of sexual differentiation in zebrafish (Xu et al. 2008), but to our knowledge, this is the first example of a non-steroidogenic pharmaceutical interfering with the timing of sexual differentiation. Changes in the timing of sexual differentiation would translate into biological implications if it altered the age-of-maturation and changed the onset of reproduction (Lester et al. 2004). Morphological observations indicated the faster sexual differentiation was in part due to a greater occurrence of males at the spermatozoa stage at 45 dpf, however this still falls within the natural range of variation noted in the species.
(Takahashi 1977; Uchida et al. 2002) and visual observations indicated that sperm was yet to be abundant in the majority of impacted fish (Figure 2.3C). Furthermore, by 60 dpf, no differences were observed in the timing of sexual differentiation for all offspring and no females were found to be at a mature stage at any time point. Although a difference in timing of sexual differentiation was evident, this effect was not likely to alter the onset of reproduction.

2.4.4 Sex Ratio in F₁ Offspring of Exposed Parents

Exposure to the steroidogenic pharmaceuticals EE2 (in Japanese medaka, Oryzias latipes, Scholz and Gutzeit 2000 and zebrafish, Örn et al. 2003, 2006; Xu et al. 2008), 17α-methyltestosterone (MT, in zebrafish, Fenske and Segner 2004; Örn et al. 2003) or the anti-estrogen fadrozole (nile tilapia, Oreochromis niloticus, Kwon et al. 2000; and zebrafish, Fenske and Segner 2004) during the period of sexual differentiation has been found to lead to male or female-biased sex ratios and even complete masculinization or feminization, depending on the drug and dose. In aquaculture, MT has been used to control sex determination (Feist et al. 1995; Van den Hurk et al. 1989). Although parental exposure studies with pharmaceuticals have not examined differences in sex ratios, changes in sex ratios have previously been documented in subsequent generations as a result of parental exposure to compounds linked to endocrine disruption. Parental exposure to phytosterols has been shown to cause a male-biased sex ratio in the F₁ offspring of zebrafish (Nakari and Erkomma 2003), while parental exposure to TCDD was
found to cause a significant shift towards females in the $F_1$ and $F_2$ offspring of zebrafish (Baker et al. 2014). Collectively, this data suggests that both direct and parental chemical exposure may be an important environmental factor in sex determination.

In the present study, paternal exposure to CBZ led to a male-biased sex ratio in juvenile offspring but only when there was no maternal exposure. Paternal exposure to GEM led to male-biased sex ratios in juvenile offspring regardless of maternal exposure. Together, these data indicate that paternal exposure history for these two compounds is important in influencing sex determination and that parental exposure to CBZ and GEM can lead to male-biased sex ratios in zebrafish.

At 60 dpf offspring from crosses with both paternal and maternal exposure to GEM (GEMM/GEMF lineage) were only male but at 45 dpf, some females were observed with maturing perinucleolar and/or cortical alveolar growth oocytes (Figure 2.2). This suggests that the GEMM/GEMF lineage was not 100% masculinized but very strongly male-biased; sample sizes for histological analyses were small enough ($n = 30–32$) that it was possible to randomly select all males.

The lineages with male-bias at the juvenile stage were not always identical to those found with male-bias at sexual maturity, even though these animals were sampled from the same cohort in the same experiment. Male-bias was found in juveniles with paternal exposure to CBZ alone (CBZM/CTLF lineage). In contrast, male-biased sex ratios were observed in sexually mature offspring after paternal CBZ exposure regardless of
maternal exposure (CBZM/CBZF and CBZM/CTLF lineages; Fraz and Wilson, unpublished data). Despite the GEMM/GEMF lineage showing the strongest male-biased sex ratio of all offspring at the juvenile stage, sexually mature adults of the same lineage did not have a biased sex ratio (Fraz and Wilson, unpublished data). Yet, offspring of paternal, but not maternal GEM exposure (GEMM/CTLF lineage) had a male-bias at sexual maturity (Fraz and Wilson, unpublished data) and in juveniles. The reason for the lack of concordance in sex ratio at juvenile and adult stages is unclear and the result was surprising.

Differences in the sex ratio of lineages between juvenile and adult stages could partially be accounted for by the much larger sample size for adults ($n = 244–354$) compared to histological assessment of juveniles. Larger sample sizes should result in a better estimation of sex ratio. It is important to note that the methods used to determine sex at each life stage differed. Sex was initially determined in adults by physical examination of secondary sex characteristics at 6 months, with sex ratio later confirmed by examining the gonads during dissections at 10 months. The histology of the adult gonads has not been examined. Secondary sexual characteristics are typically sufficient to identify sex in zebrafish; visual inspection of the gonads at dissection confirms sex in almost all cases (Wilson et al., unpublished data). In studies where all three methods (secondary sexual characteristics, visual inspection of gonads, and histology) have been used to determine sex in the same animals (e.g. Galus et al. 2013a,b), there have not been discrepancies in the sex assigned. Thus, it is unlikely that methods alone account for the differences in sex bias at the juvenile versus adult stage. This would suggest that a change
in sex was observed for some offspring between 60 dpf and 6 months, including a male to female transition for some fish.

Biased sex ratios have recovered or reversed in zebrafish after direct chemical exposures in the sexual differentiation period following rearing in clean water (Fenske et al. 2005; Xu et al. 2008). Xu et al. (2008) found a female-biased sex ratio (80% maturing ovaries with cortical alveolar growth) in zebrafish exposed to EE2 at 2 ng L$^{-1}$ from 0 to 90 dph, but these differences were no longer apparent by 180 dph after rearing in clean water for 90 days. Likewise, Fenske et al. (2005) observed 100% females (maturing/mature ovaries) in zebrafish with exposure to 3 ng L$^{-1}$ of EE2 from 0 to 118 dpf, but at 176 dpf, following rearing in clean water, 26% of fish had developed testes with spermatozoa. Although these studies demonstrate the potential for a difference in sex ratio between juveniles and adults of the same lineage, the mechanism of conversion for males to females is unclear, given that zebrafish have a natural mechanism to transform an ovary into a testis, and the existing studies document the recovered fish transitioning from a female phenotype to male phenotype (Fenske et al. 2005; Xu et al. 2008).

2.4.5 Conclusion

This study found that paternal exposure to CBZ resulted in a significantly faster sexual differentiation and a male-biased sex ratio in the juvenile F$_1$ offspring, with a potential protective effect from maternal exposure; while combined paternal and maternal exposure to GEM resulted in a significantly faster sexual differentiation and paternal exposure to GEM caused male-biased sex ratios. The sex ratios in the juvenile
offspring were not necessarily indicative of sex ratios in sexually mature adults of the same lineage, suggesting a sex reversal, including a male to female transition, took place in some fish past the juvenile sexual differentiation period. Future parental exposure studies should continue to examine reciprocal crosses to better elucidate the impact of chemical stressors as a result of paternal or maternal effects. Together, this study demonstrates that pharmaceuticals, which do not have a direct mechanism to influence or interact with steroid production, have the ability to disrupt sexual differentiation in the F₁ offspring of exposed parents.

**ACKNOWLEDGMENTS**

The authors would like to thank Mary Ellen Cybulski for training on the microtome and for H&E staining. We would also like to thank Dr. Ben Bolker, Dr. Jonathan Dushoff, and Dr. Ian Dworkin for advice on the design of the statistical analysis. Funding was provided by the Natural Science and Research Council of Canada in the form of a Discovery Grant to J.Y.W. K.M.H. was supported by an Ontario Graduate Scholarship.
### TABLES

**Table 2.1.** Morphological stages used to classify the sexual differentiation of zebrafish (*Danio rerio*) from serial sections stained with hematoxylin and eosin.

<table>
<thead>
<tr>
<th>Morphological Stage</th>
<th>Gonad Type</th>
<th>Sex Classification</th>
<th>Description¹</th>
</tr>
</thead>
</table>
| Germ cells                              | Indifferent¹        | Undifferentiated   | • Round/oval shaped cells  
• Large diameter and nucleus  
• Cytoplasm weakly stained and occupies only small fraction of cell  
• One to multiple nucleoli |
| Perinucleolar oocytes (po) juvenile     | Juvenile ovary      | Undifferentiated   | • Presence of at least one perinucleolar oocyte                               |
| Perinucleolar oocytes (po) maturing     | Maturing ovary      | Female             | • Larger gonad relative to juvenile ovary  
• Presence of well-differentiated perinucleolar oocytes  
• Inter-individual variation with the amount of perinucleolar oocytes present but relatively equal spacing between  
• Minimal stromal cells  
• Limited germ cells at periphery and caudal/cranial ends; odd germ cells centrally located  
• Larger oocytes may be approaching cortical alveolar growth (i.e. spotted clearings amongst basophilic ooplasm) but cannot be confirmed |
<p>| Cortical alveolar                       | Maturing ovary      | Female             | • Presence of several oocytes with discernable cortical alveoli or at least one oocyte with clearly defined cortical alveoli |
| Vitellogenic                            | Maturing ovary      | Female             | • Presence of at least one vitellogenic oocyte                              |</p>
<table>
<thead>
<tr>
<th>Morphological Stage</th>
<th>Gonad Type</th>
<th>Sex Classification</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transitioning</td>
<td>Altered ovary¹</td>
<td>Male</td>
<td>• Smaller gonad than ovaries with maturing perinucleolar oocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Reduction in number of perinucleolar oocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Perinucleolar oocytes undergoing degenerative process (irregular shape,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>enhanced basophilia of ooplasm and/or off-coloured patches in the ooplasm,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>break down of the nucleus)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Oocytes isolated and/or an increase in somatic cells</td>
</tr>
<tr>
<td>Early testis</td>
<td>Immature testis</td>
<td>Male</td>
<td>• Presence of germ cells in cyst-like clusters, spermatogonia or spermatocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Isolated and/or residual bodies of disintegrating oocyte(s) may be present</td>
</tr>
<tr>
<td>Spermatids</td>
<td>Immature testis</td>
<td>Male</td>
<td>• Presence of spermatids</td>
</tr>
<tr>
<td>Spermatozoa</td>
<td>Mature testis</td>
<td>Male</td>
<td>• Presence of spermatozoa</td>
</tr>
</tbody>
</table>

¹Based on Maack and Segner (2003).
Table 2.2. The occurrence of each morphological stage by age (15 to 75 days post fertilization, dpf) in untreated zebrafish gonads. Morphological stage was determined using serial sections stained with hematoxylin and eosin (Table 2.1). For each morphological stage, the day-equivalent was calculated as a weighted mean of all occurrences using \( \bar{X} = \frac{\sum_{i=1}^{N} w_i x_i}{N} \); where \( \bar{X} \) is the day-equivalent, \( x_i \) is the time point (in dpf), \( w_i \) is the occurrence of a given morphological stage at \( x_i \), and \( N \) is the total occurrence of a given morphological stage. Po = perinucleolar oocytes.

<table>
<thead>
<tr>
<th></th>
<th>15 dpf</th>
<th>25 dpf</th>
<th>35 dpf</th>
<th>45 dpf</th>
<th>60 dpf</th>
<th>75 dpf</th>
<th>Day-Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germ cells</td>
<td>15</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>Po juvenile</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>Po maturing</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>48</td>
</tr>
<tr>
<td>Cortical alveolar</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>12</td>
<td>63</td>
</tr>
<tr>
<td>Transitioning</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>Early testis</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Spermatids</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>53</td>
</tr>
<tr>
<td>Spermatozoa</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>61</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15</strong></td>
<td><strong>16</strong></td>
<td><strong>19</strong></td>
<td><strong>20</strong></td>
<td><strong>20</strong></td>
<td><strong>20</strong></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3. Mean day-equivalent (in days post fertilization, dpf) of the timing of sexual differentiation in zebrafish F₁ offspring with parental exposure to carbamazepine (CBZ) or gemfibrozil (GEM). Histological sections of the gonads of each fish were examined for each lineage at 45 and 60 dpf and assigned a morphological stage (Table 2.1). The mean day-equivalent was determined by substituting the day-equivalent (in dpf, Table 2.2) for the morphological stage of each fish and calculating a mean for each lineage. 95% confidence interval and sample size are indicated. Letters represent statistically significant differences (p < 0.05, pairwise t-tests with Bonferroni correction where two-way ANOVA detected difference) between control (CTLM/CTLF) and lineages with CBZ (lower case) or GEM (upper case) exposed parents. CTL = control/unexposed, M= male, F= female.

<table>
<thead>
<tr>
<th>Sampling Time Point (dpf)</th>
<th>Lineage</th>
<th>Mean Day-Equivalent</th>
<th>95% Confidence Interval</th>
<th>Sample Size</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>CTLM/CTLF</td>
<td>45</td>
<td>41–48</td>
<td>30</td>
<td>a A</td>
</tr>
<tr>
<td></td>
<td>CBZM/CBZF</td>
<td>46</td>
<td>43–50</td>
<td>32</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>CTLM/CBZF</td>
<td>45</td>
<td>42–47</td>
<td>32</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>CBZM/CTLF</td>
<td>54</td>
<td>51–57</td>
<td>30</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>GEMM/GEMF</td>
<td>55</td>
<td>52–59</td>
<td>32</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>CTLM/GEMF</td>
<td>50</td>
<td>47–53</td>
<td>32</td>
<td>AB</td>
</tr>
<tr>
<td></td>
<td>GEMM/CTLF</td>
<td>48</td>
<td>44–51</td>
<td>31</td>
<td>A</td>
</tr>
<tr>
<td>60</td>
<td>CTLM/CTLF</td>
<td>59</td>
<td>57–61</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBZM/CBZF</td>
<td>59</td>
<td>57–62</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTLM/CBZF</td>
<td>58</td>
<td>56–61</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBZM/CTLF</td>
<td>60</td>
<td>58–62</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEMM/GEMF</td>
<td>61</td>
<td>61–61</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTLM/GEMF</td>
<td>59</td>
<td>56–61</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GEMM/CTLF</td>
<td>60</td>
<td>58–62</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.2. Morphological stage (% of total) of the gonads by sampling time point in days post fertilization (dpf) for untreated zebrafish. No vitellogenic oocytes were observed. U = undifferentiated, F = female, M = male, po = perinucleolar oocyte(s). 15 dpf n = 15, 25 dpf n = 16, 35 dpf n = 19, 45-75 dpf n = 20.
Figure 2.2 Morphological stage (% of total) of the gonads by lineage at 45 days post fertilization. No vitellogenic oocytes were observed. U = undifferentiated, F = female, M = male, CTL = control, CBZ = carbamazepine and GEM = gemfibrozil. Lineages refer to the pharmaceutical exposures of parental zebrafish; F\textsubscript{1} offspring were reared in clean water. From left to right for the lineages \(n = 30, 32, 32, 30, 32, 32\) and 31 respectively.
Figure 2.3. The testes of zebrafish $F_1$ offspring from control (CTL), carbamazepine (CBZ) or gemfibrozil (GEM) exposed parents on 45 days post fertilization. (A) 1 of 3 testes in CTLM/CTLF (M = male, F = female) offspring, (B) 1 of 14 testes in CBZM/CTLF offspring, and (C) 1 of 22 testes in GEMM/GEMF offspring with spermatozoa present. The marked lumen of the CTLM/CTLF contains the greatest amount of spermatozoa visually observed in a CTLM/CTLF fish. Aside from a faster differentiation on average in the CBZM/CTLF and GEMM/GEMF male offspring no other gross abnormalities were observed in the testes. Hematoxylin and eosin staining; 40x. sg = spermatogonium, sc = spermatocytes, sd = spermatids and sz = spermatozoa.
Figure 2.4. Morphological stage (% of total) of the gonads by lineage at 60 days post fertilization. No vitellogenic oocytes were observed. U = undifferentiated, M = male, F = female, CTL = control, CBZ = carbamazepine, GEM = gemfibrozil. Lineages refer to the pharmaceutical exposures of parental zebrafish; F₁ offspring were reared in clean water. From left to right for the lineages n = 32, 31, 32, 31, 32, 32 and 31 respectively.
Figure 2.5. The sex ratio of the zebrafish by lineage at 60 days post fertilization. CTL = control, CBZ = carbamazepine, GEM = gemfibrozil, M = male, F = female. Lineages refer to the pharmaceutical exposures of parental fish; F₁ offspring were reared in clean water. Paternal exposure with CBZ was found to be a significant factor (p = 0.015) in male-biased sex ratios but this was dependent upon maternal exposure (interaction p = 0.005). Paternal treatment with GEM led to male-biased sex ratios (p = 0.029). From left to right for the lineages n = 32, 31, 32, 31, 32, 32 and 31 respectively.
REFERENCES


CHAPTER 3
GENERAL DISCUSSION

Investigations within our lab found that parental exposure to carbamazepine (CBZ) and gemfibrozil (GEM) resulted in impaired reproductive functions in the F₁ offspring of zebrafish (Danio rerio; Galus et al. 2014). A follow-up multigenerational study (ongoing) was then undertaken in which chronic parental exposure to 10 μg L⁻¹ CBZ or GEM was completed and F₁, F₂, and F₃ offspring were reared in clean water to determine reproductive related effects in each generation. Lineages were generated with either one or both parents exposed to distinguish paternal from maternal exposure effects. Male-biased sex ratios were observed in the F₁ sexually mature adults as a result of paternal exposure to CBZ regardless of maternal exposure and paternal exposure to GEM when there was no maternal exposure (Fraz and Wilson, unpublished data). As a part of this multigenerational study, we examined sexual differentiation in the juvenile F₁ offspring to better understand how this process was impacted by parental pharmaceutical exposure and could lead to biased sex ratios. We found that paternal exposure to CBZ resulted in a significantly faster sexual differentiation and a male-biased sex ratio in the juvenile F₁ offspring, with a potential protective effect from maternal exposure. Combined paternal and maternal exposure to GEM resulted in a significantly faster sexual differentiation and paternal exposure to GEM caused male-biased sex ratios. Interestingly, the sex ratios of the juveniles were not necessarily indicative of the sex ratios observed in sexually mature adults, suggesting a sex reversal, including a male to female transition, occurred in some
fish past the juvenile sexual differentiation period. This study clearly demonstrates the ability of the pharmaceuticals, CBZ and GEM, to disrupt sexual differentiation in the F$_1$ offspring of exposed parents.

3.1 Potential Mechanisms

The F$_1$ embryos were never directly exposed to the pharmaceuticals, as the adults were bred and the embryos were reared in clean water. As such, there are three main ways by which the F$_1$ offspring could be impacted by parental pharmaceutical exposure: gamete quality, maternal transfer of contaminants, or epigenetics. Parental exposure could have altered the quality of the sperm and/or egg. However, embryonic viability, embryonic mortality and fertilization rates did not significantly differ between treatment groups (Fraz and Wilson, unpublished data), suggesting that gamete quality was not significantly different between treatment groups.

Maternal deposition of the drug could occur during oogenesis, resulting in exposure of the offspring during yolk reabsorption. CBZ and GEM (log $K_{ow}$ 2.45 and 4.77, respectively; [http://chem.sis.nlm.nih.gov/chemidplus/](http://chem.sis.nlm.nih.gov/chemidplus/)) are lipophilic compounds. As addressed by Galus et al. (2014), this leaves the possibility for CBZ and GEM to be deposited into the oocyte during vitellogenesis and the production of yolk. In fish, lipids are the major source of energy (Babin and Vernier 1989) and hence undergo a rapid turnover, making it possible for chemicals stored in lipids to be mobilized and transported through the blood when lipids are utilized (Tyler et al. 1998). Although deposition of
pharmaceuticals into embryos has not been studied, it has been demonstrated with highly lipophilic contaminants, such as organochlorines (log K$_{ow}$ usually $>$5, e.g. DDT = 6.91; Fisk and Johnston 1998). Furthermore, GEM has been shown to bioconcentrate in the plasma of goldfish (Mimeault et al. 2005). Maternal deposition cannot be eliminated as a potential mechanism as deposition in the embryo was not measured. However, effects on reproductive parameters have predominately been observed as a result of paternal exposure in the multi-generational study, including both this data on sexual differentiation and other endpoints not discussed within this thesis (Fraz and Wilson, unpublished data). Galus et al. (2014) found paternal exposure to both CBZ and GEM had more profound impacts than maternal exposure on reproduction in the F$_1$ offspring. Together, this evidence does not favour maternal deposition into the yolk as a mechanism in this study because offspring effects would need to be linked to maternal and not paternal exposure.

Epigenetics, i.e. changes in gene expression and phenotype that are not the result of an altered DNA sequence, could influence offspring via either egg or sperm if these changes are inherited (Baker et al. 2014). Gene expression can be altered by chromatin modifications such as DNA methylation, which prevents the expression of a gene (Andersen et al. 2013). In zebrafish, the maternal DNA methylation pattern is reprogrammed at the mid-blastula transition to match the paternal DNA methylation pattern (Jiang et al. 2013; Potok et al. 2013; Zajitschek 2014). Hence epigenetic inheritance occurs paternally in zebrafish. Although the impacts of CBZ and GEM on
epigenetics have not been studied in fish, CBZ has been linked to increases in DNA methylation in human neuroblastoma cells (Asai et al. 2013). Given that the impacts of CBZ and GEM on the F₁ offspring have predominately been observed as a result of paternal exposure, and paternal effects have continued to be observed into the F₂ generation of the present ongoing multigenerational study (Fraz and Wilson, unpublished data), epigenetics is a likely mechanism in the present study.

Sex determination and differentiation could be impacted in offspring as a result of epigenetics interfering with the expression of genes that control these processes. Although polygenic sex determination is thought to be the dominant factor, it is not yet known how many and what genes are involved with regulating sex determination (Liew and Orbán 2014). A suite of genes that have been implicated in sex determination and differentiation in vertebrates have been identified in zebrafish, including amh, ar, cyp11b, cyp19a1a, dmrt1, ff1b, ff1d, nr0b1, sox9a, and wt1a (Hofsten and Olsson 2005; Orbán et al. 2009). Likewise, the exact mechanisms by which sexual differentiation initiates is unknown, however several signaling molecules and pathways have been found to be involved (Pradhan et al. 2012; Pradhan and Olsson 2014; Rodríguez-Marí et al. 2010; Sreenivasan et al. 2014). The impacts on sex ratio would suggest that one or more of the “pro-female” signaling pathways NF-κB (Pradhan et al. 2012) and Wnt/β-catenin (Sreenivasan et al. 2014) are being interfered with, and/or the “pro-male” tumour protein 53 (tp53) mediated apoptosis (Rodríguez-Marí et al. 2010). Before our results can be properly understood on a mechanistic basis, a greater understanding of the genes
involved in sex determination and the mechanisms initiating sexual differentiation needs to be confirmed in zebrafish.

To complicate matters, although paternal effects were found to be the predominate factor, different outcomes could be observed whether the offspring was from a pairwise cross (i.e. exposed mother and exposed father) or reciprocal cross (i.e. only one parent exposed). Paternal exposure to CBZ was found to impact the timing of sexual differentiation and sex ratios in offspring, but not when offspring were generated from crosses with combined parental exposure, indicating a possible protective effect of maternal exposure. Maternal effects have been known to occur as a result of maternal hormone transfer to offspring, which occurs via the yolk. In teleosts, hormone levels in eggs have been found to correlate with maternal hormone levels (Hwang et al. 1992; McCormick 1998; Mylonas et al. 1994). In damselfish (*Pomacentrus amboinensis*), maternal-derived cortisol has been shown to impact offspring growth, while manipulated-increases in testosterone (via immersion of embryos in hormone baths) have led to increases in yolk sac size (McCormick 1998, 1999). Although sexual differentiation occurs in zebrafish following yolk depletion, sex determination has been influenced by temperature from as early as 5 to 10 hours post fertilization (Abozaid et al. 2011), suggesting sex determination can be influenced earlier than the onset of sexual differentiation. Exposed mothers had lower ovarian 17β-estradiol levels (Fraz and Wilson, unpublished data), suggesting altered maternal hormone transfer may be possible. Future
studies should continue to examine reciprocal crosses in parental exposure studies to develop a better understanding of maternal effects as a result of chemical stressors.

3.2 Environmental Factors Influencing Sex Ratios in Zebrafish

In zebrafish, sex determination is primarily a result of a polygenic system, which has the ability to lead to wide-ranging sex ratios across families (Liew et al. 2012). However, the sex ratio is typically 50:50 in zebrafish (Segner et al. 2003), similar to what we observe in unexposed fish in our facility. Within our pairwise breeding, no male and female was matched twice, resulting in 75 unique breeding pairs in the generation of each lineage. This ensured no one given genotype had a strong influence on the outcome of sex ratio. While environmental factors can play a secondary role in sex determination (Liew et al. 2012; Liew and Orbán 2014), they are unlikely to be a strong influencing factor within a laboratory setting.

Temperature impacts on sex ratio (increases in males) have typically been reported at temperatures of ≥35°C (Abozaid et al. 2011, 2012; Uchida et al. 2004), with only one study observing male-biased sex ratios at 24°C and 28°C (Villamizar et al. 2012). Most laboratories, including our own, rear fish between 26–29°C for ideal development. Within our facility, we experience a great difficulty breeding at temperatures >30°C, and other facilities have observed high mortalities of larvae starting at 37°C (Liew et al. 2012; Uchida et al 2004). Likewise, hypoxia (0.8 vs. 5.8 mg L⁻¹ O₂) has been implicated in male-biased sex ratios (Shang et al. 2006) but is an irrelevant environmental factor to laboratory
studies unless it is part of the experimental design. Although density has been implicated as an environmental factor to a limited degree (increases in males observed at 67 fish L\(^{-1}\) as compared to 17 fish L\(^{-3}\)), the response has been shown to differ greatly between families (Liew et al. 2012) and hence density may not always be an influencing factor. Growth (fast growth more females, slow growth more males, as manipulated through feeding regimes; Lawrence et al. 2008) has the potential to influence sex within a laboratory setting. Ultimately, in our study the offspring of unexposed parents had an equal sex ratio, demonstrating that the environmental conditions under which they were kept were not biasing sex ratios in any one given direction.

In our study, pharmaceuticals were found to impact sex ratio with paternal exposure to CBZ or GEM leading to male-biased sex ratios in the F\(_1\) offspring. Previously, the steroidogenic pharmaceuticals EE2 (Örn et al. 2003, 2006; Xu et al. 2008), 17α-methyltestosterone (Fenske and Segner 2004; Örn et al. 2003) and fadrozole (Fenske and Segner 2004) have been shown to impact sex ratios in zebrafish. TCDD (Baker et al. 2014) and phytosterols (Nakari and Erkomma 2003) have demonstrated the ability to alter sex ratios in zebrafish offspring of exposed parents. Together, this evidence demonstrates that chemical stressors can also be considered an environmental factor that can affect sex ratios in zebrafish. Changes in sex ratio are of concern as the sex ratio influences the structure of a population and determines the reproductive potential of a population (Penman and Piferrer 2008). An increase in the proportion of males corresponds to a
decrease in females and number of eggs available for fertilization, likely reducing the reproductive success and size of a population.

3.3 Environmental Relevance

Carbamazepine (CBZ) is a prime candidate pharmaceutical to study. CBZ has been found in wastewater treatment plant (WWTP) effluent and surface waters at concentrations as high as 6.3 µg L\(^{-1}\) (Andreozzi et al. 2003; Benotti and Brownawell 2007; Metcalfe et al. 2003a), and 0.79 µg L\(^{-1}\), respectively, with reported median surface water concentrations ranging from 0.001–0.185 µg L\(^{-1}\) (Benotti and Brownawell 2007; da Silva et al. 2011; Koné et al. 2013; Lissemore et al. 2006; Metcalfe 2003a). Studies have typically found CBZ in 49–57% of sites sampled (da Silva et al. 2011; Lissemore et al. 2006; Metcalfe et al. 2003b), and CBZ has even been detected as far as 17 km downstream of a WWTP (Brun et al. 2006).

Gemfibrozil (GEM), a fibrate lipid regulator, is another pharmaceutical of interest for investigation. GEM has been found in WWTP effluent and surface waters at concentrations as high as 1.5 µg L\(^{-1}\) (Andreozzi et al. 2003; Brun et al. 2006; Metcalfe et al. 2003a) and 0.7 µg L\(^{-1}\), respectively, with reported median surface water concentrations falling in the range of 0.012–0.066 µg L\(^{-1}\) (Brun et al. 2006; da Silva et al. 2011; Kolpin et al. 2002; Koné et al. 2013; Metcalfe et al. 2003b; Peng et al. 2008; Stumpf et al. 1999). Studies have detected GEM in 0–96% of the sites sampled (Brun et al. 2006; da Silva et al. 2011; Kolpin et al. 2002; Metcalfe et al. 2003b; Peng et al. 2008). Other fibrates, which
share a mechanism of action with gemfibrozil, have been detected in surface waters including clofibric acid (active metabolite of clofibrate; max of 0.248 µg L⁻¹), benzafibrate (max of 0.47 µg L⁻¹) and fenofibrate (average 1.23 ng L⁻¹; Brun et al. 2006; da Silva et al. 2011; Koné et al. 2013; Metcalfe et al. 2003b; Peng et al. 2008; Stumpf et al. 1999).

In the present study the parental generation was exposed to 10 µg L⁻¹ of CBZ or GEM, a concentration above that reported in the environment, even when considering chemical class loads. The dose of 10 µg L⁻¹ has consistently been used in our lab as a high dose for pharmaceutical compounds and is not intended to reflect mean concentrations directly found in the environment for most single pharmaceuticals, but reasonable maximal effluent and environmental concentrations for either single compounds or compound classes (Galus et al. 2013a,b). Initially, our lab found doses of 0.5 and 10 µg L⁻¹ of CBZ or GEM to impact reproductive parameters of directly exposed adult zebrafish (Galus et al. 2013a). With a previous study of parental exposures to CBZ and GEM (Galus et al. 2014) and our ongoing multigenerational study, 10 µg L⁻¹ was selected to first establish whether or not effects could be observed in the offspring and subsequent generations as a result of parental exposure. Indeed, we found 10 µg L⁻¹ impacted reproductive parameters (Galus et al. 2014) and sexual differentiation in the offspring of exposed parents (Fraz and Wilson, unpublished data). Given that doses of 0.5 µg L⁻¹ of CBZ has led to decreases in 11-ketotestosterone in males, while both CBZ and GEM at 0.5 µg L⁻¹ have reduced fecundity and induce histopathological changes to the ovary in zebrafish
directly exposed, it would be reasonable to conduct a future parental exposure study at a lower concentration within environmental ranges, e.g. 0.5 µg L\(^{-1}\).

3.4 Relevance of the Model Organism

Fish represent a large and diverse group of vertebrates, with more than 32,000 known species of fish (Nelson 2016). In our study, the model organism zebrafish was used to examine the impact of pharmaceuticals on sexual differentiation in the F\(_1\) offspring of exposed parents. Zebrafish are classified as juvenile hermaphrodites (Maack and Segner 2003; Takahashi 1977), first developing a juvenile ovary before it differentiates into a maturing ovary or testis. This method of sexual differentiation must be considered in determining the relevance of this data to other species of fish.

There are multiple mechanisms by which fish species undergo sexual differentiation, with gonochorism and hermaphroditism each at one end of the spectrum (for review see Devlin and Nagahama 2000). Most fish are gonochoristic, developing as males or females and remaining that sex for the duration of their mature life (Devlin and Nagahama 2000). Juvenile hermaphrodites may be undifferentiated gonochorists (like zebrafish) or secondary gonochorists, in which an all-female or intersex phase, respectively, precedes the development of the mature ovary or testis. (Devlin and Nagahama 2002; Strüssmann and Nakamura 2002). Juvenile hermaphroditism has been observed in other species including the critically endangered European eel (Anguilla; Colombo and Grandi 1996), and the commercially important masu salmon (Oncorhynchus...
masou; Nakamura 1984, valued for food and recreation) and sumatra barb (Puntius tetrazona; Takahashi and Shimizu 1983, of ornamental value). Impacts on sexual differentiation in zebrafish can be informative of how pharmaceuticals can impact other species that undergo juvenile hermaphroditism.

Differentiated gonochorists develop strictly ovarian or testicular tissue (Devlin and Nagahama 2000). It is unclear if results for zebrafish would be applicable to differentiated gonochorists. Although zebrafish develop a mature ovary or testis as juveniles, the latter requires the degeneration of juvenile ovarian tissue, and not just the development of a testis. Feasibly, if a chemical stressor impacted mechanisms in the formation of the testis, instead of or in addition to the degeneration of the ovarian tissue, the results would still be relevant to differentiated gonochorists. Within the context of our current study it is unclear what was affected on a mechanistic basis and therefore it is unknown how our results would translate to differentiated gonochorists.

Hermaphrodites have the ability to produce both mature male and female gametes at some point in their life (Devlin and Nagahama 2002). Sequential hermaphrodites will first develop as males (protrandous) or females (protogynous) before reversing sex (Devlin and Nagahama 2002). Although sequential hermaphrodites undergo a sex reversal after reaching sexual maturity of one sex, it is reasonable to assume a similar process to that in zebrafish is undertaken in protogynous fish (as discussed in Wang et al. 2007). This is supported by evidence in the literature that zebrafish can initiate the
molecular events for sex reversal from a female phenotype to male phenotype past the juvenile sexual differentiation period (Fenske et al. 2005; Xu et al. 2008). As such, results with zebrafish could be applicable to protogynous fish. Protogynous species include fish of commercial importance for their recreational, food culture and/or ornamental value, including the Asian swamp eel (*Monopterus albus*; Yeung and Chan 1987, also an invasive pest), various groupers (*Epinephelus tauvina*, Lee et al. 1995; *Epinephelus merra*, Alam et al. 2005; *Epinephelus guttatus*, Shapiro et al. 1993), and wrasses (*Thalassoma bifasciatum*, Shapiro and Rasotto 1993; *Thalassoma duperrey*, Hourigan et al. 1991). However, it must be considered that some protogynous species, such as wrasses, have socially controlled sex determination (Godwin et al. 2003), and as such the relevance of chemical stressors is unclear.

3.5 Future Directions

With this study we have shown that two common, environmentally relevant pharmaceuticals, CBZ and GEM, can alter sexual differentiation in the F₁ offspring of exposed parents. Three key findings can be noted. Firstly, effects were predominately observed as a result of paternal, and not maternal exposure. Secondly, there was a potential protective effect of maternal exposure to CBZ when combined with paternal exposure. Thirdly, lineages with a difference in the sex ratio at the juvenile stage were not necessarily those with male-biased sex ratio as adults, suggesting a sex reversal,
including a male to female transition, occurred in some fish past the juvenile sexual differentiation period.

Together, these findings raise two main questions; **A) By which mechanism(s) does paternal exposure alter sexual differentiation in offspring?** Epigenetic effects in zebrafish are transferred through the male parent and have been proposed as a potential mechanism. However, it is unclear what could account for differential effects between pairwise and reciprocal crosses. Further studies should continue to examine reciprocal crosses to better understand paternal and maternal effects as a result of chemical stressors in fish, and how maternal exposures could be beneficial. **B) Were the fish undergoing sex reversal post juvenile sexual differentiation, and if so, can mechanisms in zebrafish be reprogrammed to transform males into females?** Future studies should examine the histology of the gonads of sexually mature fish and at intermediate stages between juveniles and sexual maturity to help provide answers. Although it is certain that parental exposure to pharmaceuticals can disrupt sexual differentiation in the F1 offspring of exposed parents, there is still much left to be learned about the mechanisms of paternal and maternal effects.
REFERENCES


APPENDIX

The Appendix includes a sample calculation for the day-equivalent and mean day-equivalent as discussed in Chapter 2 under section 2.2.6 Statistical Analysis. In addition, the Appendix includes Supplemental Table S1 which provides the morphological stage of the gonad observed for each fish in the control lineage at 45 days post fertilization and its respective day-equivalent to aid in the understanding of the calculation for the mean day-equivalent.

The following is a sample calculation for the day-equivalent for the germ cells morphological stage. A full list of the morphological stages and their respective day-equivalent can be found in Table 2.2. At 15 and 25 days post fertilization (dpf), 15 and 9 individuals, respectively, were categorized in the germ cells stage; the germ cells stage was not found at any other time point. We can therefore calculate the day-equivalent for the germ cells stage according to the equation below where $\bar{X}$ is the day-equivalent, $x_i$ is the time point (in dpf), $w_i$ is the occurrence of a given morphological stage at $x_i$ and $N$ is the total occurrence of a given morphological stage. For example, the germ cells stage occurred 15 times at 15 dpf ($15 \text{ dpf} \times 15$), plus 9 times at 25 dpf ($25 \text{ dpf} \times 9$), plus 0 times at every other time point. In total the germ cells stage occurred 24 times across all the time points from 15 to 75 dpf ($N = 15 + 9$).

$$\bar{X} = \frac{\sum_{i=1}^{N} x_i w_i}{N}$$

$$\bar{X}_{Germ \ cells} = \frac{(15 \text{ dpf} \times 15) + (25 \text{ dpf} \times 9)}{24}$$

$$\bar{X}_{Germ \ cells} = 19 \text{ dpf}$$
Supplemental Table S1. The morphological stage of the gonad observed for each zebrafish in the control lineage (CTLM/CTLF) at 45 days post fertilization (dpf) with the respective day-equivalent. Fish in the control lineage are the F₁ offspring of unexposed parents. The morphological stage was determined using histological sections of each fish stained with hematoxylin and eosin. A full list of the morphological stages and their respective day-equivalent can be found in Table 2.2. The data is intended to aid in the understanding of the sample calculation for the mean-day equivalent. Po = perinucleolar oocytes.

<table>
<thead>
<tr>
<th>Fish Number</th>
<th>Morphological Stage</th>
<th>Day-Equivalent (dpf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Po juvenile</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>Po juvenile</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>Po juvenile</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>Po maturing</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>Po maturing</td>
<td>48</td>
</tr>
<tr>
<td>6</td>
<td>Po maturing</td>
<td>48</td>
</tr>
<tr>
<td>7</td>
<td>Po maturing</td>
<td>48</td>
</tr>
<tr>
<td>8</td>
<td>Po maturing</td>
<td>48</td>
</tr>
<tr>
<td>9</td>
<td>Po maturing</td>
<td>48</td>
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<tr>
<td>10</td>
<td>Po maturing</td>
<td>48</td>
</tr>
<tr>
<td>11</td>
<td>Po maturing</td>
<td>48</td>
</tr>
<tr>
<td>12</td>
<td>Po maturing</td>
<td>48</td>
</tr>
<tr>
<td>13</td>
<td>Po maturing</td>
<td>48</td>
</tr>
<tr>
<td>14</td>
<td>Po maturing</td>
<td>48</td>
</tr>
<tr>
<td>15</td>
<td>Po maturing</td>
<td>48</td>
</tr>
<tr>
<td>16</td>
<td>Transitioning</td>
<td>31</td>
</tr>
<tr>
<td>17</td>
<td>Transitioning</td>
<td>31</td>
</tr>
<tr>
<td>18</td>
<td>Transitioning</td>
<td>31</td>
</tr>
<tr>
<td>19</td>
<td>Early testis</td>
<td>41</td>
</tr>
<tr>
<td>20</td>
<td>Early testis</td>
<td>41</td>
</tr>
<tr>
<td>21</td>
<td>Early testis</td>
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<td>22</td>
<td>Early testis</td>
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<td>23</td>
<td>Early testis</td>
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<td>24</td>
<td>Early testis</td>
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</tr>
<tr>
<td>25</td>
<td>Early testis</td>
<td>41</td>
</tr>
<tr>
<td>26</td>
<td>Spermatids</td>
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<td>Spermatids</td>
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<tr>
<td>28</td>
<td>Spermatozoa</td>
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</tr>
<tr>
<td>29</td>
<td>Spermatozoa</td>
<td>61</td>
</tr>
<tr>
<td>30</td>
<td>Spermatozoa</td>
<td>61</td>
</tr>
</tbody>
</table>
The following is a sample calculation for the mean day-equivalent for the control lineage (CTLM/CTLF) at 45 days post fertilization (dpf). To shorten the notation of the written equation, the equation has been notated with the day-equivalent multiplied by the number of times its respective morphological stage was observed, rather than with the straight addition of each observation. A full list of the mean-day equivalent for each lineage can be found in Table 2.3. The morphological stage of the gonad observed for each fish and its respective day equivalent for the control lineage at 45 dpf can be found in Supplemental Table S1. Based off of this data we can calculate the mean day-equivalent according to the equation below where \( \bar{X}_L \) is the mean day-equivalent for a lineage and \( N \) is the total number of fish in the lineage. For example, for the control lineage at 45 dpf the numerator is the po juvenile day-equivalent times the number of observations \( (31 \text{ dpf} \times 3) \), plus the po maturing day-equivalent times the number of observations \( (48 \text{ dpf} \times 12) \), plus the transitioning day-equivalent times the number of observations \( (31 \text{ dpf} \times 3) \), plus the early testis day-equivalent times the number of observations \( (41 \text{ dpf} \times 7) \), plus the spermatids day-equivalent times the number of observations \( (53 \text{ dpf} \times 2) \), plus the spermatozoa day-equivalent times the number of observations \( (61 \text{ dpf} \times 3) \). The denominator \( (N = 30) \) is because there were 30 fish analyzed for the control lineage at 45 days post fertilization.

\[
\bar{X}_L = \frac{\sum_{i=1}^{N} \text{Day Equivalent}}{N}
\]

\[
\bar{X}_{\text{Control Lineage}} = \frac{(31 \text{ dpf} \times 3) + (48 \text{ dpf} \times 12) + (31 \text{ dpf} \times 3) + (41 \text{ dpf} \times 7) + (53 \text{ dpf} \times 2) + (61 \text{ dpf} \times 3)}{30}
\]

\[
\bar{X}_{\text{Control Lineage}} = \frac{1338 \text{ dpf}}{30}
\]

\[
\bar{X}_{\text{Control Lineage}} = 45 \text{ dpf}
\]