CARBAMAZEPINE & GEMFIBROZIL AFFECT ZEBRAFISH REPRODUCTION

LONG TERM ADVERSE EFFECTS OF CARBAMAZEPINE AND GEMFIBROZIL ON MALE ZEBRAFISH (*Danio rerio*) REPRODUCTION

By:

Shamaila Fraz, M.Sc

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TITLE: Long Term Adverse Effects of Carbamazepine and Gemfibrozil on Zebrafish (*Danio rerio*) Reproduction

AUTHOR: Shamaila Fraz, M.Sc. (McMaster University)

SUPERVISOR: Dr. Joanna Y. Wilson

CO-SUPERVISORS: Dr. Glen Van Der Kraak (University of Guelph), Dr. Ana R. Campos (McMaster University), Dr. Rosa Da Silva (McMaster University)

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LAY ABSTRACT

Human pharmaceuticals reach aquatic environments through municipal wastewater. The bioactivity of pharmaceuticals at low concentrations has raised concerns about undesired effects in aquatic species like fish, which can experience chronic exposures. This thesis examined adverse reproductive effects of direct chronic exposure of carbamazepine and gemfibrozil to parental zebrafish and their un-exposed offspring for multiple generations. Exposure to both compounds reduced androgens and reproduction and altered behaviour, and sperm quality in males. Effects persisted in the unexposed offspring. Parental carbamazepine exposure impacted multiple generations. We suggest that carbamazepine and gemfibrozil may reduce male reproductive fitness by reducing male sex steroids.

ABSTRACT

Pharmaceuticals are emerging surface water contaminants, and are manufactured, used, and released into environment in considerable amounts. Concerns have been raised due to the inherent potency and bioactivity of these molecules, which makes effects at low concentrations more likely. The ubiquitous presence and stability of pharmaceuticals brings up concerns about the frequency and length of exposures. However, the distribution and fate of these compounds in surface water bodies is not clear. There is limited information about the potential effects in non-target, especially aquatic, species vulnerable to cumulative or lifelong exposures. Carbamazepine (CBZ) and gemfibrozil (GEM) are two of the most frequently detected pharmaceuticals in surface water. This thesis examined sub-lethal adverse reproductive effects of chronic direct exposure of CBZ and GEM to F_0 zebrafish and several generations of unexposed offspring; the effects of exposure on testicular steroidogenesis were also examined. Chronic exposure of zebrafish to CBZ and GEM reduced ex vivo production of 11KT in testes. In vivo, CBZ decreased reproductive output, 11-ketotestosterone (11KT), male courtship and aggression behaviours, and sperm morphology in F_0 parents. The F_1, F_2 and F_3 offspring of CBZ exposed males had lower reproductive output, altered courtship, aggression, sperm morphology and lower 11KT compared to fish from the unexposed lineage. The adverse effects persisted into the F_3 generation which suggested transgenerational paternal effects. GEM decreased reproductive output in F_0 parents and a reduction in 11KT, altered male courtship, aggression and sperm morphology. Unexposed F_1 male offspring, but not other generations, had sub-lethal toxic effects from parental exposure. We therefore suggest that CBZ and GEM act as endocrine disruptors in fish and that chronic exposure may reduce male reproductive fitness.

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LIST OF ABBREVIATIONS

PPCPs	Pharmaceuticals and Personal Care Products
APIs	Active Pharmaceutical Ingredients
BCF	Bioconcentration Factor
CBZ	Carbamazepine
GEM	Gemfibrozil
CTL	Control
^{o}C	Degree Celsius
µg/L	Microgram per Litre
µm	Micrometers
µS	Microsiemens
ANOVA	Analysis of Variance
Ko/w	Octanol Water Partition Coefficient
BAF	Bioaccumulation Factor
11KT	11-Ketotestosterone
WWTPs	Waste Water Treatment Plants
PPARα	Peroxisome Proliferation Activated Receptor-alpha
PPARβ	Peroxisome Proliferation Activated Receptor-beta
PPARγ	Peroxisome Proliferation Activated Receptor-gamma
EDCs	Endocrine Disrupting Chemicals
GnRHs	Gonadotropin Release Hormones
GTH	Gonadotropin Hormones
17,20βP	17α,20β-dihydroxy-4-pregnen-3-one
17,20βS	17α,20β,21-trihydroxy-4-pregnen-3-one
LC50	Lethal Concentration 50%
EC50	Effect Concentration 50%
MOA	Mode of Action
EE2	Ethinyl Estradiol
HPG	Hypothalamus-Pituitary-Gonadal
GTH	Gonadotropin Hormones

GnRHs	Gonadotropin Release Hormones
17,20βP	17α,20β-dihydroxy-4-pregnen-3-one
17,20βS	17α,20β,21-trihydroxy-4-pregnen-3-one
LH	Luteotropic Hormone
FSH	Follicle Stimulating Hormone
EDCs	Endocrine Disrupting Chemical
ADME	Absorption, Distribution, Metabolism, Excrtetion
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
BPA	Bisphenol-A
F_0	Parental generation
F_1	First Filial Generation
F_2	Second Filial Generation
F_3	Third Filial Generation
MS-222	Ethyl 3-aminobenzoate methane sulfonate
CTLM/CTLF	Control Male with Control Female
CTLM/CBZF	Control Male with Carbamazepine exposed Female
CBZM/CBZF	Carbamazepine exposed Male with Carbamazepine exposed Female
CBZM/CTLF	Carbamazepine exposed Male with Control Female
GEMM/GEMF	Gemfibrozil exposed Male with Gemfibrozil exposed Female
GEMM/CTLF	Gemfibrozil exposed Male with Control exposed Female
dpf	Day Post Fertilization
hpf	Hours Post Fertilization
VAP	Angular Path Velocity
VCL	Curvilinear Velocity
VSL	Straight Line Velocity
ELISA	Enzyme Linked Immunosorbent Assay
SEM	Standard Error of Mean
DMSO	Dimethyl Sulfoxide
NaCl	Sodium Chloride
KCl	Potassium Chloride

$CaCl_2$	Calcium Chloride
$MgSO_4$	Magnesium Sulfate
HEPES	4-2-Hydroxyethyl-1-Piperazineethanesulfonic Acid
LOQ	Limit of Quantitation
LOD	Limit of Detection
MRM	Multiple Reactions Monitoring Mode
LC-ESI-MS/MS	Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry
hCG	Human Chorionic Gonadotropin
StAR	Steroidogenic Acute Regulatory protein
25-OH cholesterol	25- Hydroxy Cholesterol
cAMP	Cyclic Adenosine Monophosphate
P450-SCC	P450-Side Chain Cleavage
CYP11a	CytochromeP450-11a
CYP17a	CytochromeP450-17a
CYP11β	CytochromeP450-11β
OECD	Organization of Economic Development
COX	Cylcoxygenease
SHBG	Sex Hormone Binding Globulin
ER	Estrogen Receptor
RXR	Retinoid X Receptor
CYP19b	Brain form of aromatase
HSD-17β3	17beta-Hydroxysteroid dehydrogenase type 3
HSD-11β2	11beta-Hydroxysteroid dehydrogenase type 2
HPA	Hypothalamus- Pituitary-Adrenal
miRNAs	Micro RNAs
tsRNAs	tRNA derived small RNAs

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis is arranged in a sandwich format approved by McMaster University and with the permission of the supervisory committee. The thesis consists of five chapters. Chapter 1 describes background information on the presence of carbamazepine and gemfibrozil, a brief overview of the current aquatic ecotoxicological concepts and state of knowledge are discussed. Chapter 2 and 3 give respective accounts of negative adverse effects of carbamazepine and gemfibrozil on parental zebrafish reproduction and the testing of maternal and paternal derived effects in the offspring. Chapter 4 describes the effects of chronic carbamazepine and gemfibrozil exposures on production of male sex steroid in zebrafish testes ex vivo. Lastly, Chapter 5 gives an overview of the results and a practical relevance of the study.

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: TRANSGENERATIONAL EFFECTS OF CHRONIC CARBAMAZEPINE EXPOSURE IN ZEBRAFISH *(Danio rerio)*

Authors: Shamaila Fraz, Abigail H. Lee, Adam Khalaf, Krishna Srinivasan, Abhilasha Vermani, Ephraim David, and Joanna Y. Wilson.

Comments: S.F. planned and conducted this study and wrote the manuscript under the supervision of J.Y.W. A.L. helped with exposures, most of sperm quality and behavioural experiments. A.L., K.S., A.V., and E.D. assisted in scoring videos of male aggression in the four generations of fish. A.K. assisted in sperm quality experiments in one generation.

CHAPTER 3: PARENTAL GEMFIBROZIL EXPOSURE IMPACTS ZEBRAFISH F_1 OFFSPRING BUT NOT SUBSEQUENT GENERATIONS

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CHAPTER 4: GEMFIBROZIL AND CARBAMAZEPINE DECREASE STEROID PRODUCTION IN ZEBRAFISH TESTIS (*Danio rerio*)

Authors: Shamaila Fraz, Abigail H. Lee, and Joanna Y. Wilson.

Comments: S.F. planned and conducted this study and wrote the manuscript under the supervision of J.Y.W. A.L. helped with collection of tissue and conducting the ex vivo tissue culture.

CHAPTER 5: GENERAL DISCUSSION

CHAPTER 1

GENERAL INTRODUCTION

1.1 PHARMACEUTICALS IN THE ENVIRONMENT

Pharmaceuticals and personal care products (PPCPs) comprise a large class of chemicals, including human and veterinary medicines used for the diagnosis, management, and treatment of disease; and chemicals used in cosmetics, personal care and hygiene products (Ellis, 2006; Corcoran et al., 2010). Since the 1990s, technological advancements in analytical chemistry and availability of instruments for detection of trace levels of pharmaceuticals has resulted in increasing reports of these micro pollutants in aquatic environments and less commonly in terrestrial environments (Corcoran et al., 2010; Santos et al., 2010; Daughton & Ternes, 1999; Hughes et al., 2013; Lapen et al., 2008). Concerns have been raised due to the inherent potency, bioactivity, ubiquitous presence, stability, unknown distribution and fate of these compounds in surface water bodies (Daughton & Ternes, 1999; Fent et al., 2006).

In the field of ecotoxicology, pharmaceuticals are classified as emerging contaminants of concern. This is mainly because active pharmaceuticals are highly potent nonpolar molecules (molecular wt. 200–500 Da), specifically designed to diffuse across biological membranes to produce biological effects and resist hydrolysis and bio-degradation (Fent et al., 2006; Kümmerer, 2010). There is limited information about the potential effects of these bioactive compounds in non-target species, especially aquatic species vulnerable to cumulative, long term or lifelong exposures (Corcoran et al., 2010;

Fent et al., 2006; Kümmerer, 2010). A high degree of conservation among molecular drug targets across various species has been indicated by several studies (Hugget et al., 2003; Brown et al., 2014; LaLone et al., 2013; 2014). Therefore, pharmaceuticals are called "agents of subtle change" due to the propensities to produce predictable adverse effects in aquatic species through conserved molecular targets/pathways and un-predictable effects by unknown mechanisms (Daughton & Ternes, 1999).

Pharmaceuticals are manufactured in hundreds of tons and have considerably large per capita consumption in industrialized countries (Whitacre et al., 2012; Fent et al., 2006; Zhang et al., 2008), which is expected to increase further due to changing demographics, increase in life expectancy, and a rise in living standards (Kümmerer, 2010). The improper disposal of pharmaceuticals is taken an important factor for environmental risk assessment; since humans not only excrete active pharmaceuticals in urine and feces, but population surveys in the UK and USA indicated disposal of sizable amounts of un-used medicines in household trash and down the drains via sink and toilet (Bound & Voulvoulis, 2005). Thus, active pharmaceutical ingredients (APIs) and metabolites have been detected in landfills (Lapen et al., 2008); waste water from hospitals, manufacturing, and municipalities; surface water such as rivers, lakes, streams (reviewed by Santos et al., 2010; Hughes et al., 2013), estuaries (Benotii & Brownawell, 2007), and coastal lagoons (Moreno-González et al., 2016); and ground and drinking water (Daughton, 2010; Jones et al., 2005). The most frequently occurring APIs belong to the pharmacological classes of antibiotics, analgesics and anti-inflammatories, beta blockers, anti-epileptics, lipid regulators, synthetic hormones, and anti-depressants

(Kostich et al., 2014; Gavrilescu et al.,2015; Fatta et al., 2007). The final concentrations of specific APIs are governed by factors like consumption patterns, extent of metabolism in human body, removal efficiency through municipal waste water treatment, degradation rates in water (photolysis, biodegradation), and partitioning between water and sediment (Daughton & Ternes, 1999; Corcoran et al., 2010; Zhang et al., 2008). APIs were typically found in European and North American treated municipal waste water effluents at 1–10 µg/L and from 0.001–1 µg/L in surface water (Fent et al., 2006; Gavrilescu et al., 2015).

Municipal waste water treatment plants (WWTPs) provide a major portal of entry for pharmaceuticals into surface water, which serve as a final sink (Sedlak & Pinkston, 2011; Fent et al., 2006). Many pharmaceuticals display pseudo-persistence in surface water because the rates of continual release into the environment surpass the rates of removal by waste water treatment and degradation by biota and UV light (Daughton & Ternes, 1999). The removal and fate of pharmaceuticals in conventional WWTPs depends on the susceptibility to complete biodegradation/mineralization and adsorption to suspended solids via hydrophobic and electrostatic interactions (reviewed by Santos et al., 2010). Biotransformation during the treatment process is not always effective because it might activate a prodrug or a conjugated metabolite (Santos et al., 2010; Sedlak & Pinkston, 2011; Fent et al., 2006). Most of the APIs or metabolites are hydrophilic in nature, fail to partition to suspended solids, and are introduced into surface water through the aqueous phase (Jones et al., 2004; Sedlak & Pinkston, 2011; Crane et al., 2006). For the removal of the residual pharmaceuticals, and to make water suitable for drinking,

additional treatment methods like ozonation, activated carbon treatment, and membrane filtration have been suggested effective (Fent et al., 2006; Daughton, 2010). Constraints to these processes include high costs, inefficient removal of some pharmaceuticals, and unknown toxicity and fate of ozonated products (Stackelberg et al., 2004; Daughton, 2010).

Pharmaceuticals with octanol/water partition coefficient (Log $K_{o/w}$) greater than 5 can bioconcentrate; however those having Log $K_{o/w}$ 3–5 are regarded lipophilic enough to bioaccumulate (Brown et al., 2007; Howard & Miur, 2011). Several studies described the bioconcentration and bioaccumulation of pharmaceuticals in fish and other aquatic organisms (Ramirez et al., 2007; Garcia et al., 2012; de Solla et al., 2016; Mimeault et al., 2005; Zenker et al., 2014; Brown et al., 2007). Bioaccumulation is the increase in concentration of a chemical in organs or tissues of an organism via uptake from the surrounding environment through all routes including absorption through skin, respiration, and food; whereas bioconcentration is the accumulation of a chemical in the aquatic organism from water only (Zenker et al., 2014; Arnot & Gobas, 2006). Bioconcentration factor (BCF) is the ratio between the concentrations of a chemical in an organism to the concentration in water at equilibrium conditions and is determined in lab under controlled conditions. The bioaccumulation factor (BAF) represents the same ratio as BCF but is typically determined in field collected animals to take into account all of the total concentration of chemicals from various routes of exposure (Arnot & Gobas, 2006).

Ecotoxicological data is available only for 1% of more than 4000 known human pharmaceuticals (Sanderson et al., 2004; Boxall et al., 2012). Most of the early ecotoxicological evaluations focused on acute toxicity (Santos et al., 2010); however since 1980s, chronic exposure of aquatic organisms has been recognized as more environmentally realistic (Crane et al., 2006; Schmitt et al., 2010). The unintended effects of APIs have been tested in non-target aquatic organisms like bacteria, algae, daphnia, cnidarians, mollusks and fish (reviewed by Vasquez et al., 2015; Schmitt et al., 2010). Nonetheless, investigation of potential chronic adverse effects in fish is considered imperative owing to evolutionary conservation of molecular drug targets across vertebrates and unknown capabilities to metabolize APIs in vivo (Huggett et al., 2003; Gunnarsson et al., 2008; Crane et al., 2006; Corcoran et al., 2010). This thesis focused on the chronic toxicity of carbamazepine and gemfibrozil in zebrafish, two commonly detected APIs in waste water effluents across the globe with mean detection frequencies of 85% and 45.3% respectively (as reviewed by Hughes et al., 2012).

1.1.1 Carbamazepine

Carbamazepine (CBZ) is a neuroactive drug structurally related to tricyclic antidepressants, primarily indicated for partial seizures in patients of all age groups, but also effective for management of bipolar disorder, neuropathic pain, and aggression due to dementia (Ambrosio et al., 2002; Bazinet et al., 2006; Freymann et al., 2005). CBZ has a multi target mode of action in humans but the exact mechanism is unknown. Its anti-epileptic action is mainly because of decreased frequency of repetitive action potentials in excitatory neurons through inhibition of voltage gated sodium channels and to a lesser

extent through antagonizing voltage gated L-type calcium channels (Ambrósio et al., 2002). Moreover, it causes an increase in extracellular levels of serotonin and dopamine transmission in brain (Ambrósio et al., 2002; Ahmad et al., 2005; Beakley et al., 2015).

Despite extensive metabolism in patients and excretion of ≤ 5% of the drug as parent compound (Gavrilescu et al., 2015), CBZ is widespread and pervasive in surface water (Zhang et al., 2008). It is poorly removed (< than 10%) through municipal waste water treatment, (Zhang et al., 2008; Andreozzi et al., 2003; Kostich et al., 2014; Lissemore et al., 2006; Gavrilescu et al., 2015), resistant to biodegradation (Zhang et al., 2008; Yan and Song, 2014) and photo degradation with a half-life of nearly 100 days at 50° latitude in winter (Andreozzi et al., 2003). Due to the ubiquity and persistence, CBZ was proposed as a tracer of organic contaminants in surface water (Gasser et al., 2011) and a marker of anthropogenic contamination in ground water (Clara et al., 2004).

The global occurrence of CBZ in municipal WWTP effluents and in surface waters was reviewed by Zhang et al. (2008). In a European survey, CBZ was detected in all municipal WWTP effluents and most of the river samples tested with 90-percentile values of 3700 ng/L and 820 ng/L respectively (Ternes, 1998), and a maximum concentration of 2100–6300 ng/L (Ternes, 1998; Andreozzi et al., 2003). In a similar US study, CBZ was found in 48 out of 50 municipal WWTP effluents at 140–460 ng/L (Kostich et al., 2014). The maximum concentrations measured in Canadian municipal WWTP effluents were 2300 ng/L (Metcalfe et al., 2003) and 3287 ng/L (Koné et al., 2013). In a survey of European river water, the maximum level of CBZ was reported to be 11561 ng/L (Loos et al., 2009). CBZ levels in surface water were 16.2–700 ng/L in

Canada (Lissemore et al., 2006; Koné et al., 2013). It was detected in finished drinking water in USA at 1–6 ng/L, (Daughton, 2010) and in Canada to a 24 ng/L maximum (Jones et al., 2005).

CBZ was shown to bioconcentrate and bioaccumulate in fish tissues (Koné et al., 2013; Ramirez et al., 2007). Valdés et al. (2014) observed a concentration dependent bioconcentration and determined a BCF equal to 0.7 and 0.9 in whole body of mosquito fish (*Gambusia affinis*) in 96 hour exposures to 10 and 100 µg/L CBZ. Others documented a BCF of 1.5–7.1 in various organs and tissues of channel catfish (*Ictalurus punctatus*), and bluntnose minnows (*Pimephales notatus*); and BAF of 2.5–3.8 in tissues of wild caught tilapia (*Oreochromis niloticus*, Garcia et al., 2012). CBZ exposure affected normal development and growth rate in medaka (*Oryzias latipes*) and zebrafish embryos (*Danio rerio*, Qiang et al., 2016; Nassef et al., 2010a; Pruvot et al., 2012). Exposure to CBZ caused oxidative damage in brain and liver of rainbow trout (*Oncorhynchus mykiss*, Li et al., 2010a; 2010b), decreased swimming speed and produced behavioural alterations in adult medaka (Nassef et al., 2010b), pumpkin seed sunfish (*Lepomis gibbosus*, Brandão et al., 2013) and *Jenynsia multidentata* (Calcagno et al., 2016). Direct acute exposure of carp sperm (*Cyprinus carpio*) reduced sperm velocity and produced oxidative damage (Li et al., 2010c). Long term CBZ exposure reduced fecundity and plasma 11-ketotestosterone (11KT, the predominant male sex hormone) in parental male zebrafish, coupled with increased incidence of apoptotic and atretic oocytes in ovaries and histological changes in kidneys of both males and females (Galus et al., 2013). The unexposed F_1 offspring had reduced fecundity in crosses involving males with paternal

exposure history and F_1 males had altered sperm swimming velocity, sperm morphology, and courtship behaviour (Galus et al., 2014).

1.1.2 Gemfibrozil

Gemfibrozil (GEM) is a non-halogenated fibrate hypolipidemic drug. It is a PPARα receptor ligand in mammals, which improves the balance of plasma lipids by decreasing triglycerides and low density lipoprotein cholesterol and increasing high density lipoprotein cholesterol (Cunningham et al., 2010; Zimetbaum et al., 1991; Chinetti et al., 2000). PPARα regulates the uptake of lipids, transformation into Acyl-CoA esters, and metabolism in peroxisomes and mitochondria (Chinetti et al., 2000). Nonetheless, GEM has been shown to exert effects independent of PPARα activation such as anti-inflammatory effects (Roy & Pahan, 2009).

GEM is included in the list of pharmaceuticals with highest use worldwide (Yan & Song, 2014). Besides extensive metabolism (~70%) in patients and excretion of ≤ 2% of the drug as parent compound (Zimetbaum et al., 1991), GEM is widespread and pervasive in surface water (Andreozzi et al., 2003; Santos et al., 2010; Fent et al., 2006). In general, GEM is inefficiently but variably (10–75 %) removed by municipal WWTPs due to differences in available secondary treatment technologies (Fent et al., 2006; Lishman et al., 2006). It resists biodegradation (Whitacre et al., 2012) and the environmental half-life estimate ranges between 119.5–288.8 days (Araujo et al., 2011). The municipal WWTP effluent concentrations in North America and Europe range between 480–2300 ng/L and 840–4760 ng/L respectively (Koné et al., 2013; Metcalfe et

al., 2003; Lishman et al., 2006; Kostich et al., 2014; Andreozzi et al., 2003). Maximal occurrence in freshwater systems was 580 ng/L in Canada (Koné et al., 2013); 790 ng/L in USA (Kolpin et al., 2002), 7780 ng/L in Spain (Muñoz et al., 2009) and 710 ng/L in Sweden (Bendz et al., 2005). It was detected in finished drinking water in USA and Canada at 0.4–1.0 ng/L (Daughton, 2010) and 70 ng/L (Jones et al., 2005), respectively.

In a nationwide US survey, GEM was found in wild caught fish (Ramirez et al., 2007). Bioconcentration was reported in goldfish (BCF = 113; Mimeault et al., 2005) and rainbow trout (BCF = 63; Brown et al., 2007). Exposure caused embryonic malabsorption syndrome in zebrafish (Raldúa et al., 2008), modified the activity of metabolic enzymes in carp liver (*Cyprinus carpio*) in vitro (Thibaut et al., 2006), reduced total plasma lipoproteins in rainbow trout (Prindiville et al., 2011), and induced antioxidant defense system in goldfish liver (*Carassius auratus*, Mimeault et al., 2006). GEM reduced plasma testosterone in goldfish (Mimeault et al., 2005), and fecundity in fathead minnows (*Pimephales promelas*, Skolness et al., 2012). Chronic exposure of adult zebrafish resulted in lower fecundity, increased atretic and pre vitellogenic oocytes in ovaries, and histological changes in kidneys (Galus et al., 2013). F_1 offspring of zebrafish, raised in clean water, had lower embryo production when there was paternal GEM exposure history. The F_1 male offspring showed impacts on courtship behaviour, sperm swimming velocity, and sperm morphology (Galus et al., 2014).

1.2 REPRODUCTIVE ENDOCRINE DISRUPTION IN FISH

Endocrine disrupting chemicals (EDCs) include a variety of chemicals like industrial chemicals, phytoestrogens, pesticides, natural hormones and pharmaceuticals (Arcand-Hoy & Benson, 1998). EDCs can interfere with the endocrine system at various levels, by altering the synthesis, storage, release, distribution, action and metabolism of hormones, or mimicking hormone action by binding to the receptors, or acting as antagonist to normal hormones; and can also cause adverse effects in the offspring of exposed organisms (Mills & Chichester, 2005). Among pharmaceuticals with known modes of action (MOA) for reproductive endocrine disruption include estrogens (ethinylestradiol, diethyl-stilbesterol), androgens (methyl testosterone, trenbolone), antiestrogens (tamoxifen, clomifene), anti-androgens (vinclozolin, flutamide), and aromatase inhibitors (ketoconazole, flutamide, fadrozole; reviewed by Mills & Chichester, 2005 and Knacker et al., 2010). However, many compounds disrupt fish reproduction through indirect mechanisms like clofibrate, which interferes with sex steroid synthesis by altering cholesterol availability, and ibuprofen through increased production of estradiol (Runnals et al., 2007; Han et al., 2010). Finally, EDCs may interfere with the HPG axis feedback regulation, and lead to neuroendocrine disruption, e.g. fluoxetine reduces isotocin and increases dopamine in goldfish brain (Mennigen et al., 2010; 2011).

Reproductive endocrine disruption in fish living downstream of municipal WWTPs has been widely reported; and often manifested as skewed sex ratios, intersex, vitellogenin induction in males, delayed gamete maturation, and reduction in gonad size

and quality of gametes (Ankley & Johnson, 2004; Sumpter, 2005). Fish are not considered to be more sensitive to EDCs than other vertebrates; but might be more susceptible in certain critical windows of life cycle (Van der Kraak et al., 2001; Ankley & Johnson, 2004). Moreover, fish inhabit contaminated water and can experience high exposures due to fast respiration and osmoregulation (Van der Kraak et al., 2001) with uncertain rates of metabolism. Fish embryos could experience high exposure by maternal deposition in eggs (Van der Kraak et al., 2001; Corcoran et al., 2010; Ostrach et al., 2008).

The fish reproductive endocrine system share basic similarities with mammals, yet have some important differences (Tokarz et al., 2013). Reproduction is controlled extrinsically by the hypothalamus–pituitary–gonadal (HPG) axis. The hypothalamus communicates with the brain and the pituitary through neuropeptides (e.g. neuropeptide-Y), gonadotropin release hormones (GnRHs) and neurotransmitters (e.g. dopamine; Le Page et al., 2011; Zohar et al., 2010). There are two GnRHs in most fish, which regulate the synthesis and release of two kinds of gonadotropin hormones (GTH) from the pituitary i.e. GTHI (luteotropic hormone-LH like) and GTHII (follicle stimulating hormone-FSH like; Zohar et al., 2010; Archand-Hoy & Benson, 1998). Contrary to mammals, dopamine inhibits the basal and GnRH stimulated release of pituitary gonadotropins in some teleost fish (Dufour et al., 2005). GTHII regulates the early and mid stages of gamete development and provides constitutive control for sex steroid biosynthesis; while GTHI controls the acute steroidogenic response needed for maturation of gametes, ovulation and spermiation (Archand-Hoy & Benson, 1998). Sex steroids

regulate gametogenesis, sexual behaviour, gonad development, secondary sex characters and sex differentiation; and provide feedback regulation (Tokarz et al., 2013). Like mammals, estradiol is the main estrogen in fish. Unlike mammals, 11KT is the most potent androgen in most fish, and 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP), and 17α,20β,21-trihydroxy-4-pregnen-3-one (17,20βS) are the dominant fish progestins (Kobayashi et al., 2002; Tokarz et al., 2015).

1.2.1 Zebrafish as a Model to Study Reproductive Endocrine Disruption

Testing of EDCs in fish models is deemed appropriate from the standpoint of ecological relevance and to produce data useful in ranking of risk to other fish species for predictive toxicity due to the high degree of conservation of potential drug targets (receptors, proteins and enzymes) across vertebrates (Ankley & Johnson, 2004; Tokarz et al., 2013; LaLone et al., 2014; Fent et al., 2006). For testing of EDCs, zebrafish is one of the three most commonly employed model species in addition to fathead minnow and Japanese medaka (Ankley & Johnson, 2004; Segner, 2009; Hutchinson et al., 2003; Tokarz et al., 2013). Extensive use of zebrafish is due to technical advantages like high fecundity, year round spawning, suitability for partial and full life cycle tests, histological screening, fully sequenced genome, capacity to apply forward and reverse genetics, development of transgenic fish, and presence of biomarker responses (McGonnell & Fowkes, 2006; Segner, 2009; Ankley & Johnson, 2004; Hutchinson et al., 2006). Some advantages directly employed in the current study are listed as follows. Due to high fecundity and a short generation time, zebrafish is regarded a suitable model for multigenerational experiments (Scholz & Mayer 2008; Hill et al., 2005; Segner, 2009).

There is significant understanding of steroid biosynthesis (Tokarz et al., 2013; 2015) and regulation, and feedback of HPG axis (Chen et al., 2010) to complement the testing of EDCs. It possesses orthologues to 86% of human drug targets (Gunnarson et al., 2008), so existing mammalian knowledge about pharmaceutical MOA and adverse effects could be useful in comprehending the effects in fish (Segner, 2009). Finally, zebrafish behaviour is extensively studied and full encyclopedic compendium of zebrafish behaviour is available (Kalueff et al., 2013).

1.2.2 Behaviour –An Emerging Tool to Study Toxicity of EDCs

The unequivocal importance of behaviour in stress ecology has given rise to increasing application in aquatic toxicology (Gerhardt, 2007; Brodin et al., 2014). Behaviour is the integrated response of an organism to internal (physiological, biochemical) and external stimuli (e.g. social cues, contaminants), which describes the relationship between two organisms or a group (Gerhardt, 2007). Alterations in aggression, locomotion, boldness, parental care, reproductive behaviour, predatory response, predator avoidance, and migration can affect a population by altering individual fitness directly and affecting higher levels of biological organization through indirect effects (Cohn & MacPhail, 1996; Brodin et al., 2014). Some well-known advantages of behavioural tests in toxicological studies include “early warning” signals of chemical exposure, higher sensitivity than survival, noninvasive testing, low cost and easy manipulations, ecological relevance, and production of valuable information on species–species interaction (Gerhardt, 2007; Hellou, 2011; Ewald, 1995).

The plasticity of behaviour provides the basis to measure the reaction of an organism to environmental changes including contaminants like EDCs (Gerhardt, 2007; Söffker & Tyler, 2012; Tokarz et al., 2015). In contrast to the classic measures of mortality (lethal concentration 50%–LC50, and effect concentration 50%–EC50) in toxicological assays, behaviour serves as a measure of sub-lethal toxicity (Brodin et al., 2014; Cohn & MacPhail, 1996), which is an advantage highly relevant for EDC exposures (Ankley et al., 2007). The applicability of various behavioural endpoints to EDC exposures in terms of sensitivity was suggested to be equivalent to biomarker responses like induction of vitellogenin and spiggin production (Söffker & Tyler, 2012). Hence, behavioural endpoints have been widely employed in testing of endocrine disrupting pharmaceuticals like fluoxetine (Mennigen et al., 2010, 2011; Schultz et al., 2011), synthetic hormones like ethinyl estradiol (EE_2, Colman et al., 2009), and others including carbamazepine and gemfibrozil (Galus et al., 2014; Nassef et al., 2010b; Brandão et al., 2013; Calcagno et al., 2016).

1.2.3 Sperm Quality – an Indicator of Reproductive Endocrine Disruption in Male Fish

Sperm quality is the quantification of desirable morphological, structural, physiological, and genetic traits, which can describe the ability of sperm to successfully fertilize egg (Rurangwa et al., 2004; Snook, 2005). Spermatocrit (weight of sperm cells/ml milt), sperm speed, sperm count, pH, osmolality, ionic composition and metabolic enzymes with energy sources are considered parameters of milt quality as a whole. Individual sperm quality parameters include sperm morphology and the integrity

of the plasma membrane, DNA and mitochondrial membrane (Fauvel et al., 2010; Rurangwa et al., 2004; Browne et al., 2015).

EDCs can presumably affect sperm quality via interfering with the extrinsic control of spermatogenesis (LH and FSH), hormonal control of spermatogenesis by altering Leydig cell steroid synthesis, and synthesis of growth factors (e.g. IgF) for germ cell proliferation (Schulz et al., 2010). Estradiol acts on sertoli cells and plays a role in spermatogonial renewal (Schulz et al., 2010); 11KT triggers proliferation of spermatogonia (Schulz et al., 2010; Tokarz et al., 2015). Lastly, 17,20βP initiates meiosis, controls the maturation of sperm, milt volume and sperm motility (Schulz et al., 2010; Scott et al., 2010).

The specific mechanisms of action for an individual EDC on sperm quality are not fully understood (Hatef et al., 2013). However there are several reports of sperm quality impairments in fish due to pharmaceutical exposure. Roach exposed to municipal waste water effluent had intersex, sperm with reduced motility and sperm speed (Jobling et al., 2002). Colfibric acid reduced sperm counts and sperm motility in fathead minnows (Runnals et al., 2007). Carbamazepine caused a reduction in carp sperm motility and velocity (Li et al., 2010c). Dutasteride exposure of fathead minnows resulted in reduced sperm viability and sperm speed (Margiotta-Casaluci et al., 2013). EE_2 caused a decrease in milt volume, percentage of motile sperm and sperm speed in grayling (Lahnsteiner et al., 2006). Hence, sperm quality could be used as biological index of male fish reproductive health in response to contaminant exposures (Hatef et al., 2013).

1.3 CHRONIC TESTING OF PHARMACEUTICALS IN FISH

1.3.1 Toxicity Tests Using Chronic Exposures

Several research reviews indicate that chronic aquatic toxicity tests are more meaningful to determine the toxicity of pharmaceuticals in terms of ecological relevance (Corcoran et al., 2010; Daughton & Ternes, 1999; Ewald, 1995; Fent et al., 2006; Ankley et al., 2007; Crane et al., 2006). This is based on several lines of evidence. Continuous long term exposures are a norm for pharmaceuticals as they are pseudo-persistent in the environment. Pharmaceuticals are inherently potent, so that low dose exposures may perturb the normal physiology and important homeostatic controls of non-target organisms to produce sub-lethal but "subtle" adverse effects (Ankley et al., 2007; Daughton & Ternes, 1999; Nicols et al., 2011; Kidd et al., 2007). Chronic testing should be taken a regular approach for pharmaceutical testing because acute tests may underestimate the toxicity of these compounds (Schmitt et al., 2010). Population level adverse effects can be studied by chronic tests (Schmitt et al., 2010; Kidd et al., 2007). Long term testing can provide an avenue to evaluate the adverse effects based on prior knowledge of the mammalian mode of action (MOA, Brown et al., 2014; Schmitt et al., 2010), but can also be used to explore imperceptible mechanisms e.g. effects that could be mediated through interaction with multiple receptor subtypes, due to differences in tissue distribution of receptors across species or differences in metabolic capacities (Ankley et al., 2009; Daughton & Ternes, 1999; Fabri, 2015). Finally, the changes in fitness related traits like reproduction and behaviour can help to understand the "subtle" long term effects of pharmaceuticals (Daughton & Ternes, 1999; Fent et al., 2006; Brodin

et al., 2014). On account of the above, potential pharmaceutical EDCs could suitably be tested at whole organism level through chronic tests and because of a focus on sub-lethal toxicity, throughout the life, a major part of life, or during sensitive windows of life cycle (early development, sexual differentiation, and active reproduction; Ankley et al., 2007). These tests commonly use fish reproduction as an endpoint, which is a highly sensitive and ecologically relevant indicator (Ewald, 1995; Overturf et al., 2015); and generate data on population level endpoints like fecundity and breeding success for ecological risk characterization (Ewald, 1995; Archand-Hoy & Benson, 1998; Ankley et al., 2007).

1.3.2 Use of Mammalian Data for Toxicity Assessment

A considerably high degree of conservation of human drug targets in aquatic vertebrates is documented by several studies (Brown et al., 2014; McRobb et al., 2014; Gunnerson et al., 2008). The appropriateness of applying mammalian mode of action and adverse effects data (called biological read across) for chronic toxicity investigation or risk prioritization in fish is highlighted by numerous studies (Hugget et al., 2003; Ankley et al., 2007; Länge & Dietrich, 2002; Arnold et al., 2014; Schmitt et al., 2010). The large body of knowledge available about pharmaceuticals due to extensive mammalian testing could be utilized in designing experiments as a first point of investigation in fish and in selecting appropriate endpoints for toxicity testing (LaLone et al., 2013; 2014; Ankley et al., 2007; Arnold et al., 2014; Fent et al., 2006; Länge & Dietrich, 2002). This approach although valid, has limitations like sparsity of data about pharmacokinetics (absorption, distribution, metabolism and excretion) in fish, poor knowledge about function of conserved receptors, differences in species sensitivity and effects mediated through

receptors or pathways not reported in mammalian literature (LaLone et al., 2014; Länge & Dietrich, 2002; Crane et al., 2006).

1.3.3 Importance of Multi/Trans-generational Effects Monitoring

"Epigenetics is the study of the processes that underlie developmental plasticity and cell differentiation and can bring about persistent developmental effects in both prokaryotes and eukaryotes" (Jablonka & Raz 2009). The epigenome is a multitude of chemical compounds that provide the means to tell the genome which genes should be expressed, can dynamically respond to environment during embryonic development, cell differentiation, and during life of an organism to produce morphological or physiological changes which may persist, and can be inherited (Jablonka & Raz 2009; Casati et al., 2015). The changes in the epigenome could occur through various mechanisms including alteration in DNA methylation, histone and chromatin modifications, and small non-coding RNAs (reviewed by Casati et al., 2015; Tchurikov, 2005; Heard & Martienssen, 2014).

Multigenerational effects (physiological or behavioural) are "context/exposure dependent", transient, defined by phenotypic changes in the offspring without affecting the genotype, and are limited to the environmental experience of the parents (reviewed by Schwindt et al., 2015; Youngson & Whitelaw, 2008). These effects occur due to direct or continuing exposure and last as long as the exposure endures. Transgenerational epigenetic effects occur when the parents transmit a change in the epigenome through gametes to the offspring (Skinner, 2011; Nilsson & Skinner, 2015), which persists in the

absence of exposure for several generations by escaping the major reprogramming events during early embryonic development (Youngson & Whitelaw, 2008). Transgenerational effects can manifest as alterations in offspring physiology, development, endocrinology, and behaviour (Schwindt, 2015; Crews, 2008).

Multi and transgenerational effects of contaminants in fish are plausible in effluent dominated water bodies, but are not incorporated in most lab studies due to the time and cost involved (reviewed by Schwindt, 2015 and Groh et al., 2015). Such effects are likely since fish present enhanced sensitivity to EDCs exposures in certain stages of life cycle, like early embryogenesis, sex determination, sex differentiation and active reproduction (Jobling & Tyler, 2003; Van der Kraak et al., 2001). Fish can deposit contaminants in eggs through bioconcentration or biomagnification and the early embryos may face exposures due to limited metabolism and excretion (Jobling & Tyler 2003; Ostrach et al., 2008), leading to delayed effects which can persist in the unexposed offspring in the absence of direct exposures (Hutchinson et al., 2005; Ostrach et al., 2008; Jobling et al., 2002). EDCs may cause transgenerational effects, such that exposure effects could be transferred to unexposed offspring, from parental exposure alone (Vandegehuchte & Janssen, 2011; Schwindt, 2015; Head et al., 2012; Casati et al., 2015).

After the late 1990s, the need for multi and transgenerational effects monitoring in fish has often been cited (Ternes & Daughton 1999; Vandegehuchte & Janssen, 2011; Groh et al., 2015), and several studies have been published since then. For example, adult benzo[a]pyrene exposure caused transgenerational effects on embryos morphology

(Corrales et al., 2014); and embryonic exposure to methyl mercury led to learning impairments in adult zebrafish (Xu et al., 2016). Transgenerational effects in fish due to exposure of mothers, fathers or both parents to estrogens, estrogen mimics, and PCBs have been reported (reviewed by Schwindt et al., 2015). A few examples include the maternal effects of EE_2 in viviparous species (Rasmussen et al., 2002), parental effects of EE_2 in zebrafish (Nash et al., 2004) and rainbow trout (Brown et al., 2007), and paternal effects of CBZ (Galus et al., 2014). After fertilization, the zebrafish sperm epigenome stays stable in early developmental stages and plays a dominant and competitive role during the major reprogramming events (Hacket & Surani, 2013; Jiang et al., 2013; Potok et al., 2013). This is reinforced by two recent studies indicating paternal transgenerational effects; where F_2 offspring of zebrafish exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) had reduced reproductive success in the exposed male lineage (Baker et al., 2014) and zebrafish males exposed to bisphenol-A (BPA) had F_2 offspring with heart deformities (Lombó et al., 2015).

1.4 RATIONALE AND HYPOTHESIS

The adequacy of chronic toxicity tests and use of fish models to understand the effects of EDCs on reproduction, and their advantages in predictive toxicology is accredited (Fent et al., 2006; Ankley et al., 2007, 2009; Crane et al., 2006; Scholz & Mayer 2008; Vestel et al., 2016). We considered long term exposure and selected the compounds (CBZ and GEM) and test concentration based on earlier studies of negative reproductive effects in F_0 zebrafish (Galus et al., 2013) and their F_1 offspring (Galus et

al., 2014); and the presenece in surface water at levels close to the our test concentration (Loos et al., 2009; Muñoz et al., 2009). Moreover, we focused on male reproductive effects of direct exposure considering the published descriptions of negative effects of CBZ and GEM in fish and mammals (Galus et al., 2013; Mimeault et al., 2006; de Oliva & Miraglia, 2009; Andretta et al., 2014). The effect on 11KT production in testes was considered in view of direct inhibitory effects of these drugs on sex steroid production in fish (Cameron, 2011) and rat testes in vitro (Liu et al., 1996; de Oliva & Miraglia, 2009; Kühn Velten et al., 1990).

We subjected the F_0 parental zebrafish to a long term exposure and evaluated the F_1–F_3 generations in the absence of exposure, acknowledging the fact that wild fish could experience long term exposure from the effluent water and negative effects may prevail due to parental exposure history (as reviewed by Schwindt et al., 2015; Corrales et al., 2014, Xu et al., 2016). These studies were designed to assess multi/transgenerational effects due to exposure to the pharmaceutical compounds chosen. With expsoure of F_0 mothers, maternal deposition is a plausible route of exposure (Ostrach et al., 2008); such that F_1 offspring with F_2 germline would be exposed (Nilsson & Skinner, 2015). Thus, the effects noted in F_1 and F_2 generations could be "context dependent" multigenerational effects (Youngson & Whitelaw, 2008); and the effects in F_3 could be considered transgenerational effects. With exposure of F_0 fathers, the effects noted in F_2 could be taken as transgenerational effects (Schwindt et al., 2015); as the possibility of accumulation of contaminants in sperm is generally considered minimal. The consideration of paternal effects was attributed to the male derived multigenerational

effects of identical exposure in unexposed F_1 zebrafish noted previously (Galus et.al, 2014) and other studies with zebrafish (Lombó et al., 2015; Baker et al., 2014).

Hence, in this thesis, I hypothesized that chronic direct exposure to CBZ and/or GEM would adversely affect parental zebrafish reproduction, male reproductive physiology and behaviour; and adverse effects of parental exposure to CBZ and/or GEM could be transmitted to unexposed male offspring. Furthermore, I hypothesized that one possible mode of adverse effects in directly treated males could be reduced gonadal 11KT synthesis which could be studied by employing ex vivo testis culture.

1.5 OBJECTIVES

The main objectives were as follows:

- To study the effects of chronic F_0 exposure of CBZ and GEM on zebrafish reproduction by analyzing embryo production, breeding success, courtship behaviour, male aggression, sperm quality parameters and sex steroid levels in vivo.
- To determine the effects of chronic CBZ and GEM exposure on production of 11KT in zebrafish testes ex vivo
- To determine if chronic parental exposure to CBZ and/or GEM can lead to adverse effects in unexposed male offspring reproduction

1.6 CHAPTER SUMMARY

Chapter 1 describes background information on the presence of CBZ and GEM in the environment and the current knowledge about release and fate of pharmaceuticals in various water bodies. A brief overview of exposure effects in aquatic species with accounts of bioconcentration and bioaccumulation are provided. The importance of long term and multi/transgenerational adverse effect testing and the utility of behaviour as an important ecotoxicological tool are discussed.

Chapter 2 gives an account of negative adverse effects of CBZ on F_0 zebrafish reproduction. Specific lineages were established by breeding F_0 control fish with exposed male or female, to determine the maternal and paternal derived effects in the offspring, The reproductive endpoints studied in F_0 were followed in F_1–F_3 generation that were reared without exposure.

Chapter 3 outlines the negative adverse effects of GEM on F_0 zebrafish, followed by the description of effects recorded in unexposed offspring from F_1 to F_3 generations; similar to chapter 2.

Chapter 4 elucidates the effects of chronic CBZ and GEM exposure on production of 11KT in zebrafish testes ex vivo. Adult fish were exposed chronically in vivo; testes explants were removed and tested with steroidogenic modulators ex vivo.

Chapter 5 gives an over view of the results, practical relevance of the study, the presumable underlying mechanisms, and future directions.

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CHAPTER 2

TRANSGENERATIONAL EFFECTS OF CHRONIC CARBAMAZEPINE EXPOSURE IN ZEBRAFISH *(Danio rerio)*

Shamaila Fraz, Abigail H. Lee, Adam Khalaf, Krishna Srinivasan, Abhilasha Vermani, Ephraim David, and Joanna Y. Wilson.

ABSTRACT

Carbamazepine (CBZ) is one of the most frequently detected pharmaceutical residues in surface water. CBZ has been reported to lower androgens in mammals and fish, and can alter the epigenome by inhibiting histone deacetylase in mammals. We investigated the hypothesis that chronic exposure of male fish to CBZ will lower androgens and that these impacts will be transmitted to unexposed male offspring. Adult zebrafish were exposed to 10 μg/L CBZ for 6 weeks, after which reproductive output was monitored. Male reproductive indices including courtship and aggressive behaviours, 11-ketotestosterone (11KT), and sperm density, morphology and swimming speed were analyzed. CBZ exposure decreased reproductive output, 11KT, male courtship and aggression behaviours, and sperm morphology in F_0 parents. Pairwise breeding generated two lines of offspring with both parents unexposed or exposed, and two lines where only one parent was exposed. Offspring from each lineage were reared in clean water. Reproductive output and male reproductive indices were assessed in F_1, F_2 and F_3 offspring to determine whether parental CBZ exposure had transgenerational impacts. The F_1, F_2 and F_3 offspring of CBZ exposed males had lower 11KT, reproductive output, and altered courtship, aggression, and sperm morphology compared to fish from unexposed lineage. Our results indicate reproductive effects to unexposed male offspring

after parental CBZ exposure and that paternal exposure history impacts the unexposed progeny up to the F_3 generations. We suggest that parental exposure to CBZ may reduce the reproductive fitness of male offspring through transgenerational effects.

2.1 INTRODUCTION

Endocrine disruption in fish has long been a point of concern in ecotoxicology. Endocrine disrupting chemicals (EDCs) are known to interfere with hormonal signaling to affect reproduction, behaviour, growth, sexual development, and immunity in fish, and to cause transgenerational effects (Pait et al., 2002; Mills & Chichester, 2005; Fent et al., 2006; Overturf et al., 2015; Daughton & Ternes, 1999; Skinner, 2014; Schwindt, 2015). Pharmaceuticals, from human and veterinary use, contaminate surface water resulting in unintentional long term exposure of non-target aquatic species and may lead to endocrine disruption and population level adverse effects in fish (Overturf et al., 2015; Kidd et al., 2007). Pharmaceuticals can cause endocrine disruption in various ways; through receptor-mediated pathways (e.g ethinyl estradiol, by acting as an estrogen receptor agonist; reviewed by Overturf et al., 2015), non-receptor mediated mechanisms (e.g. ibuprofen, by increasing production of estradiol; Han et al., 2010), and neuroendocrine actions (e.g. fluoxetine, by decreasing isotocin; reviewed by Mennigen et al., 2011). Dietary uptake, active respiration, osmoregulation and maternal depositions in eggs are major routes contributing to the uptake and bioactivity of EDCs in fish (Barber, 2008; Van Der Kraak et al., 2001). Exposures during the sensitive windows of active reproduction, sexual differentiation, and early embryonic development through contaminants deposited in

eggs, may lead to delayed effects which can persist in the unexposed offspring in the absence of direct exposures (Hutchinson et al., 2005; Ostrach et al., 2008; Jobling et al., 2002). Fish populations in a municipal waste water effluent impacted watershed could be considered more vulnerable to the exposure routes described (Mills & Chichester, 2005). However, currently there is a lack of data on multi and transgenerational effects due to the time and resource intensiveness of such studies (Ankley & Johnson, 2004; Groh et al., 2015).

Among many anthropogenic compounds found in surface waters, the neuroactive anti-epileptic drug carbamazepine (CBZ) is one of the most frequently occurring pharmaceutical residues (Zhang et al., 2008). Yet, the impacts of carbamazepine exposure on aquatic species are relatively unknown. CBZ is indicated for the treatment of epilepsy, bipolar disorder, neuropathic pain, hypersexual behaviour and aggression due to dementia (Ambrósio et al., 2002; Freymann et al., 2005), with an estimated annual global consumption of 1014 tons (Zhang et al., 2008). It is insufficiently removed (< 10%) by conventional municipal waste water treatment processes and constantly released in wastewater, persistent in the environment, and resistant to bio and photo degradation (Zhang et al., 2008; Andreozzi et al., 2003). The maximum reported concentrations of CBZ in treated municipal waste water effluents in Europe, US and Canada were up to 6300, 460 and 3287ng/L respectively (Ternes, 1998; Kostich et al., 2014; Koné et al., 2013); whereas the maximum levels in various surface water bodies in these regions were up to 11,561, 263 and 700 ng/L respectively (Loos et al., 2009; Kolpin et al., 2004; Koné et al., 2013). CBZ was detected in finished drinking water in Canada and USA to

maximal concentrations of 24 and 6 ng/L respectively (Gavrilescu et al., 2015; Daughton, 2010). Due to the high detection frequency, incomplete removal by waste water treatment, and persistence to degradation, CBZ was proposed a tracer of organic contaminants in surface water (Gasser et al., 2011).

CBZ was predicted to be potentially hazardous in five EU countries (Ferrari et al., 2003). It was detected in wild caught tilapia (*O. niloticus*) and sunfish (*Lepomis sp.*) in USA, mosquito fish (*G. affinis*) in Argentina, greenside darter (*E.blennioides*) and fluted-shell mussels (*L. costata*) in Canada, and in mollusks (*C. glaucum*, *P. nobilis*, *M. trunculus* and *L. aurata*) in Spain (Garcia et al., 2012; Ramirez et al., 2007; Valdés et al., 2014; Wang et al., 2011; de Solla et al., 2016; Moreno-González et al., 2016). Despite frequent occurrences there is a general lack of chronic toxicity data. Among the small number of fish studies, 9 days expsoure of adult medaka (*O. latipes*) to 6.15 mg/L CBZ led to reduction in both swimming speed and feeding behaviour (Nassef et al., 2010). A 72 hour expsoure of pumpkinseed sunfish (*L. gibbosus*) to a range of CBZ concentrations from 62.5–1000 μg/L led to reduced anxiety (Brandão et al., 2013). Fourteen days exspoure of adult *Jenynsia multidentata* to CBZ concentrations from 10–200 μg/L reduced swimming speed (Calcagno et al., 2016). In vitro acute (2 hour) exposure of common carp (*C. carpio*) sperm to 2 and 20 mg/L CBZ produced oxidative damage and reduced motility and velocity (Li et al., 2010).

Treatment induced adverse effects of CBZ on human male reproductive system are well known e.g. lower serum testosterone (Isojärvi et al., 1995; Bauer et al., 2004), sperm counts and motility, and higher frequency of morphologically abnormal sperm,

(Isojarvi et al., 2004; Chen et al., 1992), decreased sexual desire, and impaired sexual function (Montouris & Morris, 2005; Reis et al., 2013). Damage to sertoli, germinal lineage cells, seminiferous epithelium, reduced serum testosterone, and increased sperm abnormalities were suggested in rats (de Oliva & Miraglia, 2009; Andretta et al., 2014). These reports laid the foundation for investigations in our lab (Galus et al., 2013; 2014), based on the fact that the mammalian mode of action and adverse effects data for chronic toxicity could be used as a starting point to assess effects in other vertebrates (Ankley et al., 2007; Huggett et al., 2003). Our lab showed that 6 weeks waterborne exposure of adult zebrafish (*D. rerio*) to both 0.5 and 10 µg/L CBZ reduced mean embryo production (Galus et al., 2013), which was confirmed for the higher dose in a subsequent experiment (Galus et al., 2014). A significant reduction in male plasma 11-ketotestosterone (11KT) occurred with 0.5 µg/L CBZ exposure; estradiol was not altered in females (Galus et al., 2013). There was increased incidence of atretic oocytes in female ovaries, with no histological changes in testes (Galus et al., 2013). Parental exposure to 10 µg/L CBZ had effects on the unexposed adult F_1 offspring; offspring showed alterations in sperm velocity, morphology and male courtship behaviour (Galus et al., 2014). Moreover, embryo production and breeding success were lower in two pairwise cross combinations; 1) when the males with parental CBZ exposure were crossed with females with identical background, and 2) when the males with parental CBZ exposure were crossed with control females (Galus et al., 2014). Since effects were not noted with offspring that were from crosses between exposed mothers and control fathers, this suggested that paternal exposure to CBZ was important for offspring effects (Galus et al., 2014).

In the current study, we hypothesized that multi or transgenerational effects could be elicited after chronic 10 µg/L CBZ parental exposure and that paternal exposure would be most important. We addressed the following questions; i) Does chronic direct exposure of F_0 parents impact multiple generations of unexposed offspring? ii) Does the individual maternal or paternal history impact the offspring equally? To answer these questions, we set up 4 lineages from the F_0 by crossing; 1) control males with control females (CTLM/CTLF), 2) control males with CBZ exposed females (CTLM/CBZF), 3) CBZ exposed males with CTL females (CBZM/CTLF), and 4) CBZ exposed males with similar females (CBZM/CBZF). We reared the offspring in clean water and followed these four lineages through F_1, F_2, and F_3 generations.

2.2 MATERIALS AND METHODS

2.2.1 General Fish Maintenance and Care

Adult wildtype zebrafish (*D. rerio*) were housed in a multi-rack housing system (AHAB, Pentair Aquatic Ecosystems Inc. USA) at a density of 3 fish per liter and 50:50 sex ratios. The housing system was equipped with automated dosing of sodium bicarbonate (Aquatic Eco systems Inc. USA) and Instant Ocean sea salts (Instant Ocean salts, Spectrum brand, USA) to distilled water; and programmed to ensure 10% water renewal per day which was validated regularly. The control monitor (YSI brand, YSI Inc. USA) was programmed to maintain temperature within a range of 27 – 29°C, pH from 6.8–8.0, dissolved oxygen 80 –100%, and conductivity at 300–400 µS, with regular validations. Measured parameters always remained within the range indicated. Fish were

kept on a 14:10 h day night cycle and were fed twice a day with tropical fish flakes (Nutrafin Max tropical fish flakes, Hagen, USA) and once a day with live adult brine shrimps (*Artemia nauplii;* Atremia Cysts, Inve aquaculture nutrition USA). All methods involving the use or handling of fish were approved by the McMaster Animal Care Ethics Review Board under the Animal Utilization Protocol # 16-09-34 to JYW.

2.2.2 CBZ Exposure of F_0

Prior to distribution of fish to treatment groups, breeding output was monitored in tanks (with 30 fish each in equal sex ratio) every day for 15 days. Embryo traps (rectangular food grade polypropylene boxes with netted lids and suitable dimensions to cover the bottom of tank) were placed into the bottom of the tank at first light and embryos collected after 1.5 hours; embryos were counted to determine reproductive output. After 2 weeks, tanks with similar reproductive output were selected and randomly distributed to treatments. Dosing solution of carbamazepine (Sigma Aldrich, Canada) was prepared in DMSO, added in exposure tanks to achieve the 10 µg/L concentration. There were triplicate tanks in each of the two treatment groups i.e. solvent CTL and 10 µg/L of CBZ, and the final concentration of DMSO (Caledon Lab Chemicals, USA) in all the tanks (CTL, CBZ) was 0.004%. Thirty fish were housed per tank with recirculating water, at a density of 3 per liter and 50:50 sex ratios (CTL, CBZ). During the 67 day exposure period, water quality was maintained through 90% change out every third day with dose renewal. Temperature and pH were monitored daily, and weekly monitoring of conductivity, dissolved oxygen (portable YSI, YSI incorporated USA), nitrate, nitrite, general hardness, carbonate hardness and ammonia were conducted (Nutrafin Test,

Hagen, USA). An overview of exposure and endpoint sampling for F_0 is provided in Fig. 2.1 A.

2.2.3 Breeding of F_0 to Set up Four Lineages

After 6 weeks of exposure, and daily monitoring of reproduction by whole tank mating, males and females were sorted by visual inspection and pairwise breeding crosses were arranged within and across treatments to get a total of four lineages i.e. (1) CTLM/CTLF (2) CTLM/CBZF (3) CBZM/CTLF and (4) CBZM/CBZF. These lineages were generated to assess the possibility of gender derived exposure effects of CBZ, as had been previously identified for F_1 only (Galus et al., 2013). There were 75 crosses in each of the four breeding combinations and no pair was crossed more than once. Breeding success was assessed based on the number of successful spawns out of a total of 75 crosses for each treatment group, and the number of embryos per spawn were counted to determine mean embryo production per female. Because of feasibility contraints, pairwise breeding assessment was completed in 17 days, arranging 32 crosses per day. Mating pairs were set up overnight and returned to the exposure tanks the next morning. Different mating combinations were selected randomly on each day to ensure that crosses from each tank and treatment group were assessed throughout the 17 day period of pairwise mating.

Embryos from breeding pairs were removed 1.5 hours after first light and rinsed with E3 embryo medium (NaCl–292 mg/L, KCl–2.83 mg/L, MgSO4–5.5 mg/L, CaCl2–5.5 mg/L), counted under a microscope, and then transferred to 120 x 20 mm glass petri

dishes at a density of 150/dish in an incubator at 28°C for 6 hours. At 6 hours post fertilization (hpf), the number of embryos was counted, including viable eggs, unfertilized eggs and non-viable (dead at 6 hpf).

The four lineages of offspring were reared in E3 embryo medium at a density of 250 in rectangular food grade polyprolylene boxes (19.5 x 11.5 x 8.5 cm) and maintained at 28°C. From 1– 5 day post fertilization (dpf), embryos underwent daily checks and dead embryos were removed. At 5 dpf, hatchlings were fed a 50:50 mixture of Grade 0 food (Hatch fry encapsulation Grade 0, Argent chemical labs, USA) and finely crushed flakes, 4 times a day with 50% daily medium renewal. From 8 dpf, the food was supplemented with live *Artemia* larvae, and 90% daily medium renewal. Between 12–16 dpf, E3 was gradually replaced with system water. At 21 dpf, the powder feed was switched to 50:50 mixture of Grade 1 food (Hatch fry encapsulation Grade 1, Argent chemical labs, USA) plus finely crushed flakes; and at 30 dpf, to 50:50 mixture of Grade 3 food (Hatch fry encapsulation Grade 3, Argent chemical labs, USA) plus finely crushed flakes. At 25–30 dpf, hatchlings were transferred to the multi rack zebrafish holding system at a density of 50 fish/3L tank and maintained at a flow rate of 75–80 ml/minute. From 35 dpf, feeding frequency was reduced to 3 times daily with powder feed in the morning and adult *Artemia* at noon. From 60 dpf, fish were shifted to coarsely crushed flake feed twice and adult *Artemia* once daily and maintained at 3fish/L density. This rearing protocol was utilized for raising embryos from each lineage of F_1–F_3 generations. Biological endpoints were analyzed in F_0 after 6 weeks of CBZ exposure and in the F_1–F_3 offspring starting at 6 months of age (Fig. 2.1B).

2.2.4 Behavioural Tests

Prior to and during pairwise breeding, fish underwent behavioural assays for aggression and courtship behaviour. One male from a treatment group was transferred to a breeding tank (Aquatic Eco systems Inc. USA; 14.5 x 9.2 x 8 cm) held in a water bath at 28°C. After habituation of fish to the tank for 10 minutes, a mirror was quickly introduced alongside the tank wall and the interaction with the mirror was videotaped using a Cannon Legria HF R56 camera for 5 minutes. Videos were analyzed blind, scoring the frequency (count) and duration of time spent in head butting/biting the mirror, parallel swimming to the mirror and freezing (Kalueff et al., 2013; nF_0 = 94–126 , nF_1 = 28–41, nF_2 = 37–39, and nF_3 = 38–42 videos per group). Parallel swimming to the mirror is an aversive display where two fish align their body head to tail, lift up the dorsal fin and extend the caudal fin (Kalueff et al., 2013). Head butting/biting characterize the fish approaching the mirror image from the front in an attempt to bite (Kalueff et al., 2013). Freezing defines the immobile condition deprived of activity except for gills and the eyes; it is a submissive response related to anxiety/fear like behaviour, boldness and stress in zebrafish (Kalueff et al., 2013).

After the mirror test, a female fish was added to the tank, with a divider to separate male and female; the tank had an insert with slits in the bottom for egg collection. All tanks were maintained overnight at 28°C. The following morning, after the first light, the dividers were removed, each pair was provided opportunity to mate for 1.5 hour and courtship behaviour was videotaped during the first 10 minutes. Male courtship displays including Chase, Nudge, Lead, Lateral and Quiver were quantified for both the

frequency and duration, as has been previously described (Darrow & Harris, 2004). Chase and nudge describe the male actively following the female with attempts of touching her body or tail, respectively (Kalueff et al., 2013). Lead describes the male swimming rapidly in tight circles or figure 8 pattern close to the female or to the same spot more than twice, so as to lead her to the spawning site (Darrow & Harris, 2004). Lateral display consists of male swimming alongside the female, with their bodies aligned; and during quiver, the male exhibits short range high frequency body oscillations which stimulate the release of eggs by the female (Kalueff et al., 2013).Videos were scored blind, using windows media player at slow speed to determine the frequency (count) and duration of displays outlined above, (nF_0 = 36–42, nF_1 = 30–42, nF_2 = 34–35, nF_3 = 39–41 videos per group). For both behavioural assays, the videos in which the fish were sedentary for more than 75% of the total time recorded were excluded from the statistical analysis.

2.2.5 Sperm Collection and Analysis

Males were separated from females overnight to facilitate milt accumulation. Closely after first light, fish were anesthetized with 0.006 mM MS-222 (Sigma Aldrich, Canada) solution at pH 7, in clean fish water until loss of equilibrium. Fish were held with ventral side upwards in a wet, soft sponge holder, the urogenital opening was blotted dry, and milt was extruded by gently squeezing the lateral sides with a blunt forceps, into a pre-weighed capillary tube. The weight of sample was converted to volume assuming a density of 1 g/ml (Dulka et al., 1987). After collection, the fish were moved to a recovery bath at 28°C. The sample sizes were 34–42 in F_0, 42–44 in F_1, 39–47 in F_2 and 25–36 in F_3 generations, respectively.

Independent sets of males were used for analysis of sperm speed and morphology. Sperm speed (n = 22 fish per group) was measured by diluting milt in 50 µl of fish water at 28°C; very quickly transferring 5 ul of this on a welled slide and recording for 60 seconds using Astro IIDC recording software with a sperm tracker system that consisted of an Olympus CX41 light microscope mounted with Prosilica EC-650 digital camera at 200x magnification. The videos were edited to 1 second intervals clips using iMovie (20–21 seconds, 30–31 seconds, 40–41 seconds, and 50–51 seconds) and played in windows media player in a loop. Sperm swimming velocities i.e. Angular path velocity (VAP) and Curvilinear velocity (VCL) were determined with Ceros CASA (computer assisted sperm analysis; n = 22 fish per group). VAP is the velocity on a computed smooth path and VCL is the actual velocity along the swimming track (Rurangwa et al., 2004). Sperm morphology (n = 25 fish per group and 20 sperm per fish) was studied by diluting milt with 50 µl of 10% neutral buffer Formalin (Fisher, Canada) mixing thoroughly, and fixing samples for 1–2 hour. A Motic BA-130 microscope with digital camera was used to image 20–25 sperm per sample at 400x magnification in 5µl of fixed sample. ImageJ software from National Institutes of Health's (available at http://rsb.info.nih.gov/ij/) was employed to measure sperm head length and width (straight line), mid-piece length and tail length (freehand line).

2.2.6 Sex Steroids

Reproductively mature male fish were terminally sampled from directly exposed F_0 and 7– 8.5 months old unexposed adult F_1–F_3 offspring, after reproductive, behavioural and sperm sampling and used for whole body 11KT assay (nF_0 = 15–18, nF_1, nF_2 = 15

nF_3 = 20 fish per group). Whole body estradiol was measured in directly exposed F_0 females only (n = 15–18). Whole body steroid assay was chosen due to the limitation of extremely small plasma sample size obtained from individual fish.The extractions were conducted by method described by Alsop & Vijayan, 2008. Fish from each tank were randomly sampled, euthanized, blotted dry, weighed, snap frozen in liquid nitrogen and then stored at -80°C. Whole fish were homogenized with 5 times w/v ice cold nanopure water. Steroids were separated in the organic phase by 3 repeated extractions of 4 volumes of diethyl ether (Fisher, Canada), using 0.5 ml of homogenate of a single fish. The ether extracts were evaporated to dryness in water bath and air dried for 2 hr. The samples were reconstituted with 0.5 ml ELISA buffer provided in the commercially available steroid hormone kit (Cayman Chemical, Ann Arbor, MI, USA). The tubes were kept at 4^{o}C for 12 h, with occasional vortexing, prior to use. The hormone was quantified in duplicates using a colorimetric 96-well ELISA kit. The extraction efficiency was between 76–80%. The experimentally determined limit of detection for 11KT analysis was 1.37 ± 0.23 pg/ml (mean ± S.D), and inter- and intra-assay coefficients of variation were less than 18% and 5% respectively. The experimentally determined cross reactivity of the ELISA kit for testosterone was 0.016 %. The mean experimental limit of detection for estradiol assay was 18.44 ± 2.35 pg/ml (mean ± S.D) and inter- and intra-assay coefficients of variation were less than14% and 4% respectively.

2.2.7 Statistics

Pairwise breeding (F_0–F_3) was determined as mean embryo production per female, analyzed by Mann Whitney rank sum test with Bonferroni correction. Breeding success

data was analyzed by Chi square test with Bonferroni correction. The data from two behavioural endpoints (courtship behaviour and aggression), and sperm morphometry (F_0–F_3) were analyzed by Kruskal-Wallis ANOVA with Dunn test. The whole body 11KT and estradiol data were analyzed using ANOVA with Holm-Sidak test. A linear regression model was used to interpret data of sperm swimming velocities; the Q–Q Plots were analyzed to confirm the assumptions of residues with normal behaviour graphically and the confidence interval was set at 95%. Statistical differences were detected through multiple comparisons with controls in F_0 (CBZ vs CTL), with control breeding pair (CTLM/CTLF), or with the control lineage in F_1–F_3 generations. Except sperm speed, all data analyses were performed using Sigma Plot version 10.0. Sperm speed data was analyzed using R version 3.2.2. All data are described as mean ± SEM wherever relevant except sperm morphology which is summarized as median (Table 2.3); and the level of statistical significance was fixed at $p \leq 0.05$, unless readjusted through Bonferroni correction ($p \leq 0.01$).

2.3 RESULTS

2.3.1 Effects of Direct Exposure of F_0

Reproduction was negatively impacted by CBZ exposure; fecundity, based on pairwise breeding crosses of CBZM/CBZF (Fig. 2.2) was significantly reduced compared to controls, regardless whether mean embryo production per female (decreased 57.4% from controls, Fig. 2.2A) or breeding success (22.7% versus 42.7% for controls, Fig. 2.2B) was used as the endpoint. There were no statistically significant differences in

unfertilized and non-viable eggs and the survival of embryos/hatchlings across treatment groups (data not shown).

CBZ exposure changed the frequency of multiple male courtship displays (Fig. 2.3) and decreased the total time spent on courtship, relative to the controls (data not shown). CBZ exposed males showed fewer chase and nudge displays, regardless of the exposure group of the female, and fewer lateral and quiver displays when crossed with CBZF (Fig. 2.3). Chase and nudge were decreased when CTL males were crossed with CBZ exposed females (Fig. 2.3).When male aggression and freezing towards a conspecific were quantified using a mirror test, CBZ exposed males presented decreased aggression as shorter time durations of parallel swims in front of the mirror (Fig. 2.4).

CBZ exposed males had sperm with smaller head diameter and tail length, and longer head and mid piece relative to the controls (Fig. 2.5) whereas milt volume and sperm swimming velocity remained unchanged (data not shown). Whole body 11KT in exposed males was significantly lower (30.4%) relative to the controls (Fig. 2.6). Whole body estradiol in exposed versus control females was not statistically different (Suppl. Fig. 2.1).

2.3.2 Effects in Adult F_1–F_3 Offspring

F_1 offspring were generated by pairwise crosses of the parental exposed and unexposed zebrafish, followed by rearing in clean water; for subsequent generations, adult male and female zebrafish were crossed within each lineage and remained unexposed (Fig. 2.1B). The F_1 offspring of the CBZM/CBZF lineage, and F_2 and F_3

offspring of the CBZM/CTLF lineage produced fewer embryos (94, 81.8 and 80.4%, reductions respectively) relative to the controls (CTLM/CTLF) offspring (Figure 2.7). In the F_1–F_3 generations, there were no statistically significant differences in unfertilized and non-viable eggs or the survival of embryos/hatchlings across treatment groups (data not shown).

The male courtship behaviour data from F_1–F_3 offspring are summarized in Fig. 2.8 and Table 2.1, showing frequencies and time durations of displays, respectively. The F_1 males from the CBZM/CTLF lineage displayed chase, nudge, lead and lateral less frequently; and those from the lineage CBZM/CBZF had fewer lead, lateral and quiver displays than the control F_1 males (Fig. 2.8A). F_2 males from two lineages (CBZM/CTLF, CBZM/CBZF) showed more leads relative to the control males (Fig. 2.8B). Furthermore the F_3 males from CBZM/CTLF lineage showed fewer chase and lead, but more nudges; whereas those from CBZM/CBZF lineage presented lead display more often as compared to the males from control lineage (Fig. 2.8C).

The results of male aggression test in F_1–F_3 generations are presented in Fig. 2.9 and Table 2.2, showing time durations and frequencies of displays, respectively. The time spent in head butting/biting the mirror image was significantly less as compared to the controls in the F_1 males from CBZM/CTLF lineage (Fig. 2.9A). F_1 males from CBZM/CBZF lineage allocated significantly less time in swimming parallel to the mirror, but more time freezing as compared to F_1 controls (Fig. 2.9A). The F_2 male offspring from the lineage CBZM/CTLF spent significantly less time swimming parallel to the mirror, head butting and freezing; while those from the lineage CBZM/CBZF spent less

time in head butting (Fig. 2.9B). The F_3 males in both lineages from an exposed F_0 father spent less time head butting and in parallel swims (Fig. 2.9C).

Three sperm quality indices (morphology, swim speed, and milt volume) were assessed in the F_1, F_2 and F_3 adult males. The sperm from F_1, F_2, and F_3 males in the two lineages with CBZ exposure in the paternal line (CBZM/CTLF and CBZM/CBZF) had smaller head diameter; F_1 and F_2 offspring had smaller mid-piece length (Table 2.3). Tail length was smaller in the CBZM/CTLF lineages in F_1, F_2 generation and the CBZM/CBZF lineage in the F_1 generation (Table 2.3). Sperm swimming speed, as measured by VCL (Fig. 2.10), was lower relative to the controls in the F_1–F_3 males from the CBZM/CTLF lineage (Fig. 2.10). Similar differences were found in VAP (data not shown). No differences in milt volume were detected in the F_1–F_3 progeny when compared to the respective controls (data not shown).

Whole body 11KT in male offspring from the CBZM/CTLF lineage was significantly reduced relative to the controls in F_1 (40.5% reduction), and F_2 generations (33.1% reduction); and in the F_1 males of CBZM/CBZF lineage (39.5% reduction, Fig. 2.11).

2.4 DISCUSSION

We monitored fecundity by pairwise breeding of chronically exposed zebrafish and detected significant declines in mean embryo production per female (Fig. 2.2). Our tank breeding data (not shown here) was similarly reduced and the percent decrease in fecundity over day 1–42 of exposure closely agree with previous research (Galus et al.,

2013; 2014) that used the same species and same compound/dosing as reported here. Likewise, 11KT was significantly decreased in whole body homogenates (Fig. 2.6), as had been previously identified in plasma (Galus et al., 2013). Although we did not verify the nominal concentrations in the water in this experiment, we have done that for multiple exposures (Galus et al., 2013; Chapter, 4). In a repeat of this exposure, with exactly same methods of stock preparation, storage, and dosing regimen, the actual water concentrations were very close to the nominal concentrations (Chapter 4). We have found, in five separate exposure experiments, consistent reproductive effects with chronic 10 μg/L CBZ exposures, which allow the use of reproduction as a biological endpoint to confirm adequate dosing of tanks. Adverse effects of parental CBZ exposure on reproduction, organ histology, and steroid levels had been previously assessed (Galus et al., 2013; 2014); impacts on aggression, courtship behaviour and sperm quality had not yet been examined in F_0 males. Yet, impacts of parental CBZ exposure on courtship and sperm in F_1 male offspring were documented (Galus et al., 2013; 2014). We examined the potential for direct exposure of CBZ to alter courtship, aggression, sperm quality, and steroids in male F_0 zebrafish. Reproduction and courtship behaviour were assessed in pairwise crosses in four combinations; by crossing male and female from the same treatment (CTLM/CTLF, CBZM/CBZF); and by crossing an exposed male or female with a control partner (CTLM/CBZF, CBZM/CTLF). Further, we reared these lineages for three generations and found persistent impacts of paternal CBZ exposure on offspring.

2.4.1 Effects of Direct Exposure of F_0

Direct exposure effects in F_0 were determined to study the context/background of anticipated effects in offspring and to test the potential endocrine disruption using a range of reproductive indices. Parental male zebrafish had lower 11KT in whole body (Fig. 2.6), analogous to the significantly lower plasma 11KT in male fish chronically exposed to 0.5 µg/L CBZ exposure; and a declining trend with 10 µg/L exposure (Galus et al., 2013). Similar effects have been largely reported in mammals. Reduced circulating testosterone in male rats (de Oliva & Miraglia, 2009; Andretta et al., 2014) and in Leydig cell cultured in vitro followed CBZ exposure (Kühn-Velten et al., 1990). 11KT is the male specific androgen in many fish, important for a wide array of reproductive processes like spermatogenesis, sexual behaviour, aggression, sexual differentiation, testicular growth, development and maturation (Tokarz et al., 2013; 2015). It is the most potent androgen and a physiologically relevant mediator of endocrine disruption in male zebrafish (Scholz & Myer, 2008).

Disturbed reproduction was evident as fecundity declined and inefficient male courtship was present in the two cross combinations including CBZ exposed males (CBZM/CTLF, CBZM/CBZF). Similar effects on male courtship were noted by EE_2 exposure of adult zebrafish males (Colman et al., 2009). The most plausible explanation of reduced fecundity in crosses including exposed males would be the reduced male ability to evoke eggs release in females, as could be inferred from scores of male courtship (Baker et al., 2014). Lower frequencies of male stimulatory displays may indicate impaired male stimulation, as these displays are repeatedly performed during

hours of active spawning (Darrow & Harris, 2004; Spence et al., 2007). Frequency of chase and nudge are positively correlated with successful courtship behaviour (Darrow & Harris, 2004). Lower frequency of lateral and quiver appeared to be linked with decreased fertilization bouts (personal observations; Fig. 2.2 & 2. 3). Altered courtship might be due to reduced pheromonal contact with the female (Kalueff et al., 2013), as in zebrafish the glucuronide and sulfated form of sex hormones act as a pheromonal inducer (Gerlach, 2006); and 11KT act as a "primer" for the manifestation of normal sexual behaviour in teleost fish (Munakata & Kobayashi, 2010). Alternatively, the potential of CBZ for direct neuro endocrine effects might be implied from reports of both the presence and bioconcentration of this compound in fish brain (Kulkarni et al., 2014; Garcia et al., 2012); although this would need experimental verification.

Despite several reports, the specific mechanisms of action of individual EDCs on fish sperm quality are not fully understood (reviewed by Hatef et al., 2013). In teleost fish, the alterations in sperm morphological traits (Fig. 2.5) without changes in swimming speed have been previously documented (reviewed by Humphries et al., 2008), which may imply lower fecundity at the cost of number or longevity (Pizzari & Parker, 2009). We were not able to determine sperm counts, as the volume of milt per fish was extremely small and accurate measurement of milt volume was challenging. In general we aimed to collect samples from fish with minimal stress and avoiding any lethal physical injuries. Moreover, due to time constraint, we could not directly assess fertilization success through male competitive or male replacement crosses. However, a small number of unfertilized eggs were observed in both pairwise cross combinations

including CBZ males while no unfertilized eggs were found in crosses with unexposed control males; which might be suggestive of lower fertilization rates. Nonetheless, experiments for estimating fertilization rates could help comprehend the differences better.

The lower aggression in CBZ exposed males support the previously established link between lower androgen and lower aggression, as assessed by a mirror test (Desjardins & Fernald, 2010; Dijkstra et al., 2012) and may indicate disturbance of social hierarchies by the exposure. Similar effects on male aggression were noted by other studies testing EE_2 in zebrafish (Colman et al., 2009; Coe et al., 2008). Androgens are associated with social hierarchies in zebrafish (Filby et al., 2010) and aggressive males can better exploit the food and have good quality sperm to gain long term fitness advantages (Ariyomo & Carter 2013; Ariyomo & Watt, 2012). Moreover, considering that a high brain serotonin in zebrafish males is associated with subordinate behaviour (Filby et al., 2010), and CBZ increases extracellular serotonin through agonistic interaction with serotonin receptors in mammalian brain (Beakley et al., 2015; Daily et al., 1998); it would be intriguing to examine this hypothetical connection in zebrafish through direct investigations employing CBZ exposure.

2.4.2 Effects in Adult F_1–F_3 Offspring

This study examined parental effects of F_0 exposure in unexposed F_1–F_3 offspring and generated lineages that included only one or both exposed parents. Like other EDCs, direct exposure of F_0 might decrease the number of desirable attributes in sperm (Dada et

al., 2012; Kime & Nash 1999), which could be transmitted to unexposed offspring through the paternal line (reviewed by Chen et al., 2016). When only the male parent was exposed, effects into the F_2 generation could be considered as transgenerational effects (Schwindt, 2015). When both parents were exposed, maternal deposition might expose F_1 offspring with F_2 germline (Galus et al., 2014) such that the effects in F_3 could be transgenerational effects and any phenotypic effects observed up to the F_2 generation would be "context dependent" multigenerational effects (Nilsson & Skinner 2015).

In the current study, there were consistent transgenerational paternal effects in the F_1–F_3 offspring from one CBZ paternal lineage (CBZM/CTLF) for most of the tested endpoints, depicting an overall disturbed reproduction. First, we noted a reduction in reproductive output of F_2–F_3 offspring from CBZ paternal lineage (CBZM/CTLF) relative to the controls (CTLM/CTLF). Similar to our results, in an experiment where F_0 zebrafish were exposed to TCDD, a transgenerational decrease in fecundity of unexposed offspring was noted up to F_2 generation, in crosses where control females were crossed with males from TCDD lineage (Baker et al., 2014). Second, the effects of F_0 exposure on male courtship relative to the control lineage were evident in the F_1–F_3 offspring from the exposed paternal lineage CBZM/CTLF (Fig. 2.8A–2.8C). The range of effects in F_1 had close similarities to those identified in the F_0 and might indicate the possibility of the imprints of paternal behaviour, as offspring can be affected by the endocrine and the social experiences of the parents (Crews, 2008; reviewed by Bohacek & Mansuy, 2015; Danchin et al., 2011). Moreover, our results integrate with former reports of reduction in nudge and lateral displays in unexposed F_1 male offspring from identical parental

exposure to CBZ (Galus et al., 2014). Third, sperm morphology (Table 2.3) was altered in males from the CBZM/CTLF lineage of F_1–F_3 generations, with reduced swimming velocity (Fig. 2.10) at 20 sec post activation. This conforms to earlier reports of alterations in morphology and velocity of sperm from F_1 zebrafish due to parental CBZ exposure (Galus et al., 2014). Lastly, lower reproductive fitness in the males from the exposed paternal lineage CBZM/CTLF could be interpreted from lower 11KT–a physiologically relevant indicator of male sexual health (Fig. 2.11); and reduced male aggression – a social indicator of reproductive success (Fig. 2.11, Scholz & Myer, 2008; Ariyomo & Watt, 2012). We take these as paternal effects because of the absence of effects in the lineage with maternal exposure alone, and manifestation in the lineage with paternal exposure alone, which indirectly support the transmission to the offspring via sperm. Moreover, for an effect to be taken as transgenerational it should persist in the unexposed F_2 offspring (Schwindt, 2015).

The paternal effects of CBZ exposure might also be suggested in the second lineage with CBZ male exposure history (CBZM/CBZF). We visualized these as paternal effects because of an absence of effects in the offspring of the lineage including maternal exposure history alone (CTLM/CBZF). The effects in the CBZM/CBZF lineage persisted to the F_3 generation in some, but not necessarily in all of the endpoints tested. Effects on fecundity and 11KT seemed to be limited to the context of exposure and presented only in the F_1 generation. Nonetheless, changes in sperm morphology, male courtship and aggressive behaviour were consistent up to F_3 generation of the CBZM/CBZF lineage.

"Epigenetics is the inheritance of the information in the DNA to express genes in space and time specific manner through mitotic or meiotic division, without modifying DNA sequences" (Casati et al., 2015). The epigenome could be modified through nutritional, behavioural, environmental and chemical influences (Danchin et al., 2011; Casati et al., 2015) and involves various mechanisms including DNA methylation, histone modifications and small non coding RNAs like (piwi RNAs, micro RNAs and t-RNA derived small RNAs; Tchurikov, 2005). Traditionally under the influence of mammalian model, mothers are implied to have a greater influence on the offspring epigenome since mothers not only provide the genetic material to the zygote, but a developing environment for the foetus; literature was dominated by the reports of maternal effects of EDCs exposure in fish. However, recently there is increasing evidence suggesting the epigenetic inheritance through paternal line (sperm) in mammals (reviewed by Chen et al., 2016; Aiken & Ozanne, 2014), and in fish (reviewed by Schwindt, 2015). The importance of studying paternal effects in zebrafish is supported by the contemporary evidence that in contrast to mammals, zebrafish sperm DNA methylome plays a dominant or competitive role during the major reprogramming events in early development and is stably inherited by the offspring germline (Potock et al., 2013; Jiang et al., 2013). Recently published literature also indicates transmission of Bisphenol-A exposure effects in unexposed zebrafish offspring through the paternal line (Lombó et al., 2015) and lower fertilization in F_2 offspring of TCDD paternal lineage (Baker et al., 2014). Furthermore, behavioural patterns modified in response to the environment are documented to pass on through paternal germline imprinting, or through "experience dependent epigenetic inheritance"

and could be responsible of similarities between parents and offspring (Danchin et al., 2011). Analogous to our results, learning impairments in F_3 zebrafish due to F_0 exposure to methyl mercury have been reported recently (Xu et al., 2016).

We recorded effects on various male reproductive indices in unexposed F_1–F_3 offspring from the lineages with history of CBZ exposure in F_0 male parents; and propose transgenerational epigenetic effects as the possible underlying mechanism. The epigenetic mechanism seems plausible considering that CBZ is reported as a histone deacetylase inhibitor and can interfere with histone modifications; histone deacetylase is involved in constriction of the histones-a transcriptionally inactive state (Beutler et al., 2005; Andretta et al., 2014; Gefroh-Grimes et al., 2016; Smith et al., 2012). We therefore suggest that CBZ might have altered the epigenetic programming of F_0 sperm during spermatogenesis (Dada et al., 2012; Schwindt, 2015), or through somatic transfer of information to sperm (Chen et al., 2016; de Oliva & Miraglia, 2009; Jablonka & Raz, 2009) resulting in paternal transgenerational inheritance (Lomobo et al., 2015).

2.5 CONCLUSIONS

This study provided evidence of endocrine adverse effects of CBZ on zebrafish reproduction in the directly exposed F_0 parents and the transmission to unexposed male offspring through the paternal line. To our knowledge this is the first study showing the transgenerational phenotypic effects of long term exposure to CBZ and one of the few studies reporting paternal effects. We indicate paternal exposure history an important determinant of adverse effects, because altered phenotype and behaviour can affect fitness

and potentially have long term consequences. In addition, this study raised many interesting questions for future examination, including the hypotheses of epigenetic changes during spermatogenesis in directly exposed F_0 and the search for the underlying mechanisms (DNA methylation, small non-coding RNAs, post translational modifications of histones and chromatin arrangement). As CBZ bioconcentrates in fish brain and is bioavailable, a better understanding of neuroendocrine effects of CBZ in zebrafish could be helpful in comprehending the physiological basis of behavioural alterations and reproductive impacts. Most of the endpoints included in the current study apparently shared a common link of reduced 11KT. However, unlike steroid estrogens, the experimental linkages between epigenetic mechanisms, testes function, reproduction and behaviour in connection to androgens in fish is needed to better understand the paradigm of paternal transgenerational effects.

2.6 TABLES & FIGURES

Table 2.1: Time durations of male courtship displays in F_0 parents exposed to 10 µg/L CBZ for 6 weeks; and unexposed offspring from F_1, F_2 and F_3 generations. Unexposed (CTL) and exposed (CBZ) males (M) and females (F) were crossed at the F_0 generation; F_1–F_3 generations were crossed within each lineage. Asterisks indicate significant differences from the respective controls, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (nF_0 = 36–42, nF_1 = 30–42, nF_2 = 34–35, nF_3 = 39–41 videos per group).

	Description of F_0 crosses OR offspring lineages	Durations of courtship displays (seconds) (Mean ± SEM)				
		Chase	Nudge	Lead	Lateral	Quiver
F_0	CTLM/CTLF	271 ± 17	35 ± 4	127 ±16	22.8 ± 4	1.38 ± 0
	CTLM/CBZF	172 ± 21*	18 ± 3*	83 ± 17	15.5 ± 4	0.83 ± 0
	CBZM/CTLF	128 ± 20*	15 ± 3*	97 ± 17	9.96 ± 3	0.10 ± 0
	CBZM/CBZF	174 ± 21*	19 ± 3*	94 ± 17	4.7 ± 2*	0.66 ± 0
F_1	CTLM/CTLF	262 ±10	21 ± 2	169 ± 12	4.4 ± 2	0.82 ± 0
	CTLM/CBZF	225 ± 23	14 ± 3	114 ± 18	3.0 ± 1	0.64 ± 0
	CBZM/CTLF	173 ± 17*	16 ± 2	90 ± 14	2.1 ± 1	0.31 ± 0
	CBZM/CBZF	194 ± 18*	15 ± 3	96 ± 18*	0.3 ± 0	0.05 ± 0*
F_2	CTLM/CTLF	187 ± 13	29 ± 4	163 ± 14	2.5 ± 2	1.18 ± 0
	CTLM/CBZF	232 ± 19	28 ± 3	163 ± 19	2.3 ± 1	0.28 ± 0
	CBZM/CTLF	179 ± 16	35 ± 4	185 ± 16	4.1 ± 1	0.36 ± 0
	CBZM/CBZF	202 ± 12	39 ± 7	152 ± 18	2.6 ± 1	0.26 ± 0
F_3	CTLM/CTLF	182 ± 13	37 ± 4	259 ± 14	2.2 ± 2	0.4 ± 0
	CTLM/CBZF	179 ± 19	39 ± 3	270 ± 19	2.0 ± 1	0.4 ± 0
	CBZM/CTLF	181 ± 16	52 ± 4*	290 ± 16	1.5 ± 1	0.3 ± 0
	CBZM/CBZF	187 ± 12	47 ± 7	282 ± 18	3.1 ± 1	0.6 ± 0

Table 2.2: Frequencies (counts) of male aggressive displays in unexposed offspring from F_1, F_2 and F_3 generations. Offspring lineages were from crosses between males (M) and females (F) from F_0 exposed (CBZ) and unexposed (CTL) parent crosses; all offspring generations were unexposed. Asterisks indicate significant differences from the respective controls, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (nF_1 = 28–41, nF_2 = 31–39, nF_3 = 30–41 videos per group).

	Description of F_0 males OR offspring lineages	Frequencies of aggressive displays (Mean ± SEM)		
		Head butt	Parallel swim	Freeze
F_0	CTLM	96 ± 3.7	35 ± 1.9	7 ± 0.7
	CBZM	91 ± 4.1	31 ± 2.3*****	7 ± 0.9
F_1	CTLM/CTLF	84 ± 3.8	9 ± 0.8	1 ± 0.3
	CTLM/CBZF	77 ± 4.6	12 ± 0.8	2 ± 0.3
	CBZM/CTLF	68 ± 3.1*****	12 ± 0.9	3 ± 0.7
	CBZM/CBZF	107 ± 8.0	21 ± 2.2*****	5 ± 1.0*****
F_2	CTLM/CTLF	75 ± 3.6	6 ± 0.3	2 ± 0.5
	CTLM/CBZF	61 ± 3.1*****	8 ± 0.5	2 ± 0.6
	CBZM/CTLF	68 ± 2.9	6 ± 0.4	1 ± 0.3
	CBZM/CBZF	64 ± 3.4	8 ± 0.4	2 ± 0.4
F_3	CTLM/CTLF	126 ± 9.0	8 ± 0.9	0 ± 0.1
	CTLM/CBZF	110 ± 6.0	9 ± 0.7	0 ± 0.1
	CBZM/CTLF	89 ± 7.5*****	10 ± 0.6	0 ± 0.2
	CBZM/CBZF	79 ± 5.5*****	10 ± 0.8	1 ± 0.2

Table 2.3: Morphometric measurements of sperm in unexposed zebrafish offspring from F_1, F_2 and F_3 generations. Offspring lineages were from crosses between males (M) and females (F) from F_0 exposed (CBZ) and unexposed (CTL) parent crosses; all offspring generations ($F_1 – F_3$) were unexposed. For morphological measures, diameter (D) and length (L) were measured for the sperm head, midpiece (MP) and tail. Asterisks indicate significant differences from the controls, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (n = 25 fish per group and 20 sperm per sample).

	Parameters of sperm morphology	Sperm Morphometrics μm (Median)				
		CTLM /CTLF	CTLM /CBZF	CBZM /CTLF	CBZM /CBZF	H-Statistic
F_1	Head D.	5.077	4.854	4.216*	4.472*	246.80
	Head L.	6.667	6.324	5.735*	6.290*	293.27
	MP. L.	3.336	2.981	2.807*	2.981*	102.20
	Tail L.	45.00	43.60	42.40*	41.20*	184.20
F_2	Head D.	5.548	5.548	5.088*	5.333*	373.94
	Head L.	6.369	6.412	6.708*	6.688*	384.40
	MP. L.	3.333	3.333	2.667*	2.535*	308.34
	Tail L.	52.95	53.18	51.44*	52.67	45.09
F_3	Head D.	4.636	4.527	4.108*	4.268*	138.53
	Head L.	5.543	5.324	5.050*	4.792*	426.33
	MP. L.	2.142	2.028	2.130	2.136	28.72
	Tail L.	67.23	67.54	72.96*	66.64	202.62

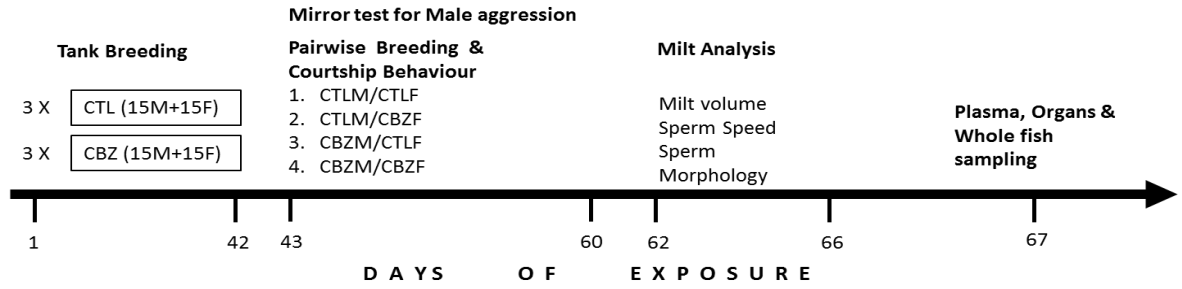


Figure 2.1A: Schematic of exposure and endpoint sampling for direct exposure of F_0 to 10 µg/L CBZ.

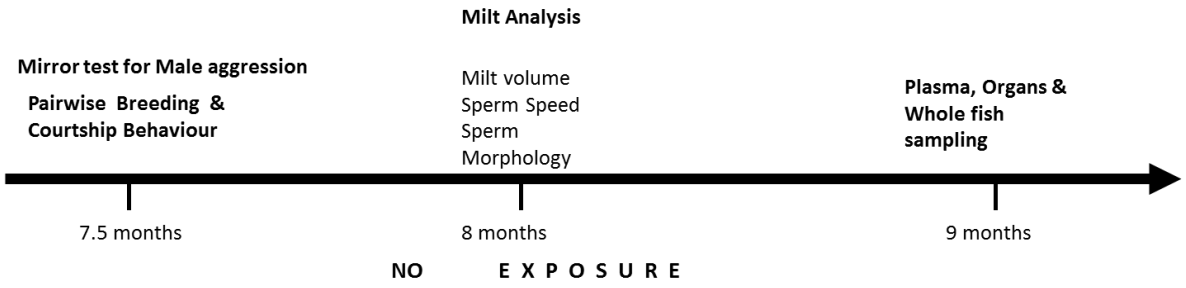


Figure 2.1B: Schematic of experiment with the offspring for each of F_1, F_2, and F_3 generations

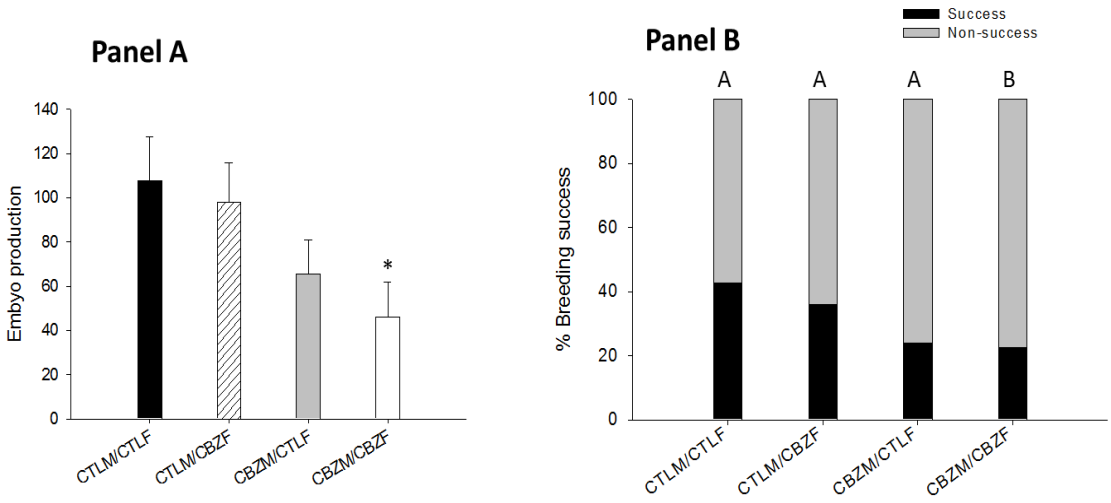


Figure 2.2: Embryo production (mean ± SEM, panel A) and breeding success (panel B) in four combinations of breeding pairs from F_0 zebrafish after 6 weeks exposure to 10 µg/L carbamazepine (CBZ). X-axes in panel A and B indicate breeding pair combinations. Control (CTL) males (M) were crossed with either CTL or exposed female (F; CTLM/CTLF and CTLM/CBZF); and CBZ exposed males were crossed with either exposed female or CTL female (CBZM/CBZF and CBZM/CTLF). Asterisk and different letters indicate significant differences from the controls. Mann Whitney rank sum test (A) and Chi square test (B) with Bonferroni correction, $p \leq 0.01$ (n = 75 crosses per group).

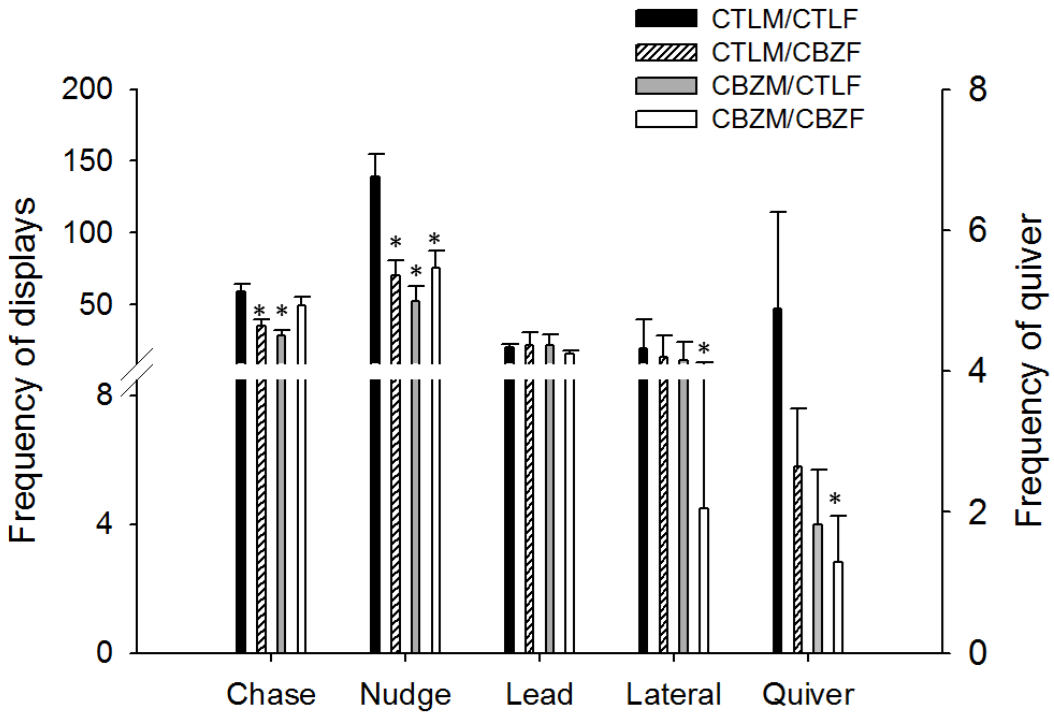


Figure 2.3: Frequencies/counts (mean ± SEM) of male courtship displays in F_0 zebrafish after chronic 6 weeks exposure to 10 µg/L carbamazepine (CBZ). Courtship videos were taken after first light in crosses from exposed (CBZ) and unexposed (CTL) fish with one male (M) and one female (F) in each tank, to generate four combinations indicated in figure legend. Videos were scored blind to determine frequency of five behaviours (see materials and methods for descriptions). Asterisks indicate significant differences from the controls, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (n = 36–42 videos per group).

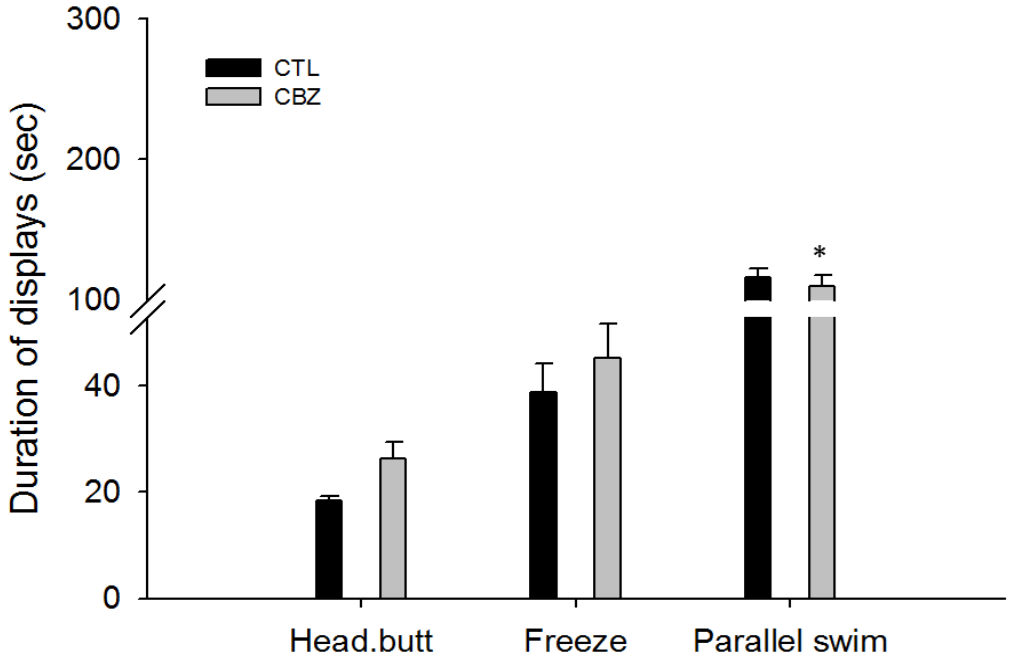


Figure 2.4: Time durations (mean ± SEM) of male aggressive (head butt, parallel swims) or anxiety (freeze) displays in F_0 zebrafish after chronic 6 weeks exposure to 10 µg/L carbamazepine (CBZ). Videos of mirror interactions were taken from exposed (CBZ) and unexposed (CTL) male fish. The videos were scored blind to determine the duration of three behaviours (see materials and methods for descriptions). Asterisk indicates significant difference from the controls, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (nCTL = 126 and, nCBZ = 94).

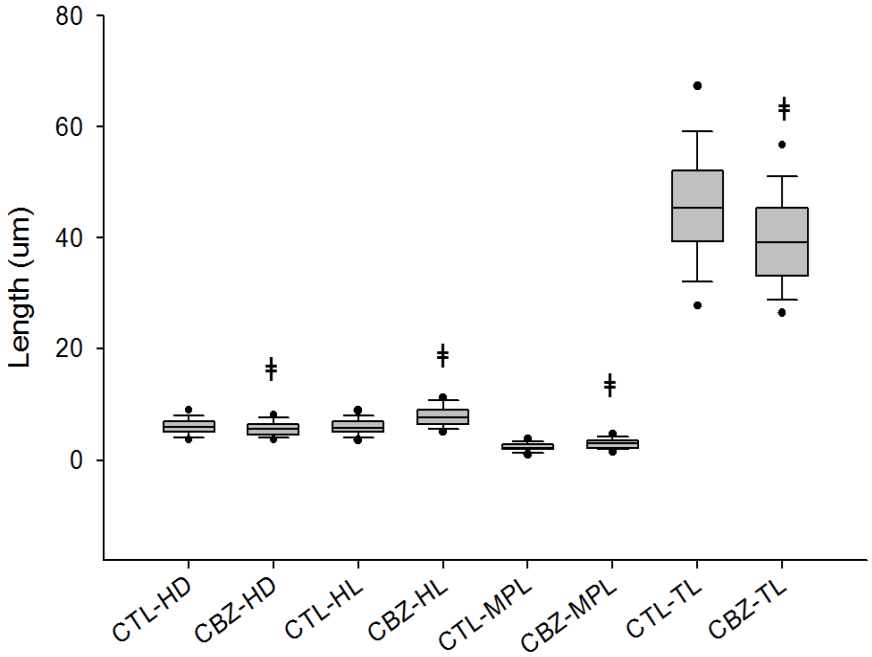


Figure 2.5: Morphometric measurements (mean ± SEM) of zebrafish sperm from F_0 fish exposed to 10 µg/L carbamazepine (CBZ). Milt samples were collected from exposed (CBZ) and unexposed (CTL) male fish, fixed in formalin, and sperm were imaged using Motic BA-130 microscope at 400 x magnification. Head diameter (HD), head length (HL), midpiece length (MPL) and tail length (TL) were quantified. Daggers indicate significant differences from the controls, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (n = 20 fish per group and 25 sperm per sample).

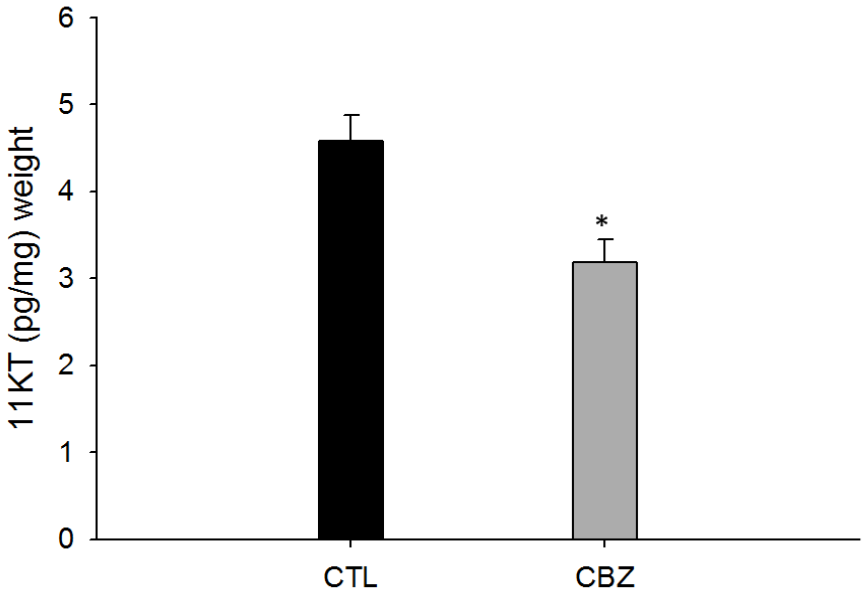


Figure 2.6: 11-Ketotestosterone (mean ± SEM) in whole body homogenates of F_0 zebrafish males from exposed (CBZ) and unexposed (CTL) groups. Asterisk indicates significant differences from the controls, ANOVA with Holm-Sidak test, $p \leq 0.05$, (nCTL = 15, nCBZ = 18).

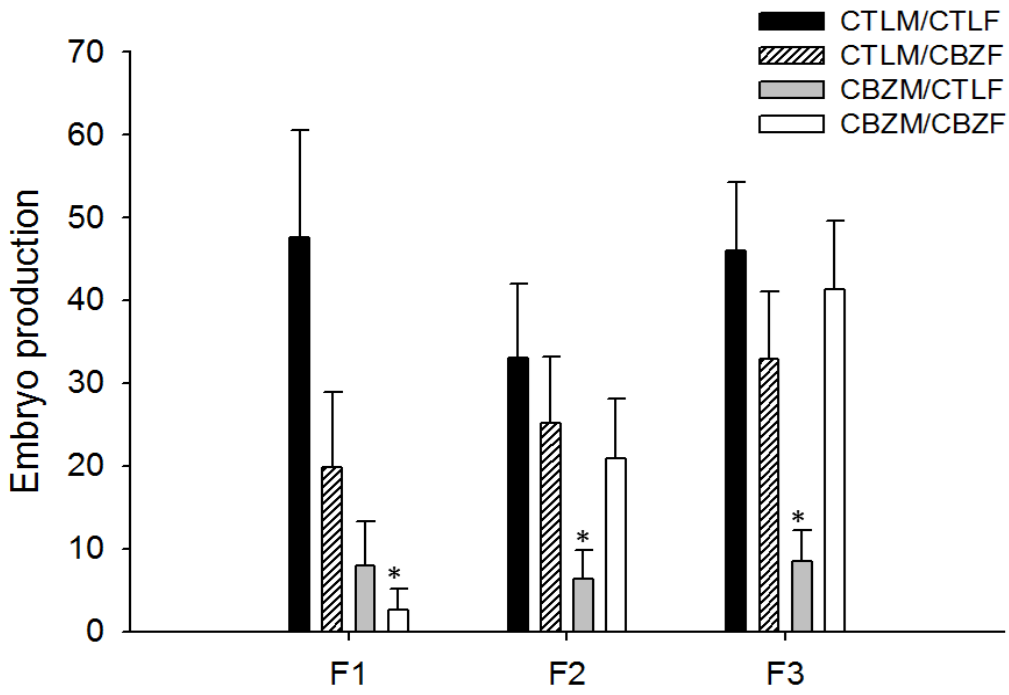
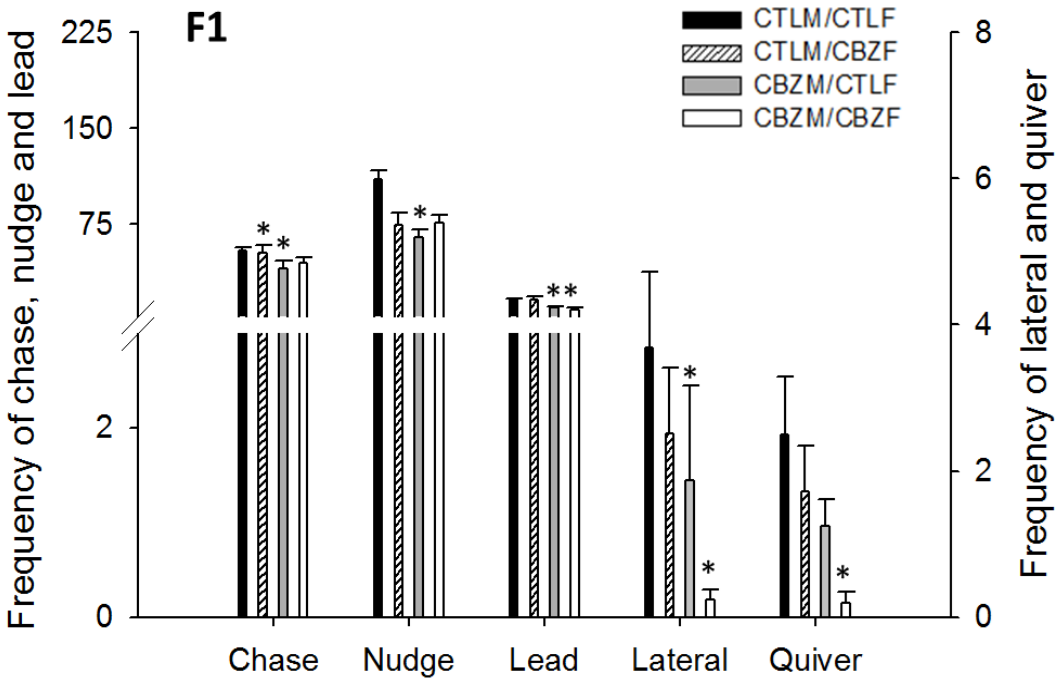
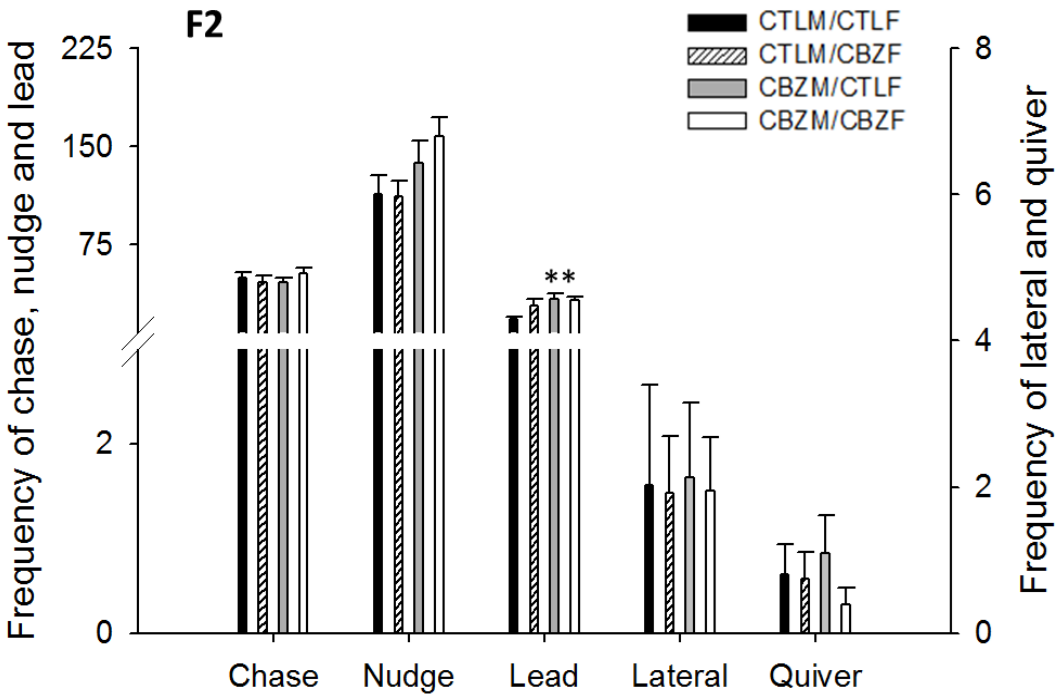


Figure 2.7: Embryo production (mean ± SEM) in breeding pairs of unexposed zebrafish offspring from four lineages of F_1, F_2 and F_3 generations. The figure legend indicates lineages generated with F_0 males (M) and females (F) from unexposed (CTL) and carbamazepine (CBZ) exposure treatments (see section 2.2.3). Pairs of male and female from the same lineage were crossed together for the F_2 and F_3 generations. Asterisks indicate significant differences from the respective control lineage within a generation, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (n = 75 crosses per group).


F1
CTLM/CTLF
CTLM/CBZF
CBZM/CTLF
CBZM/CBZF
Frequency of chase, nudge and lead
Frequency of lateral and quiver
225
150
75
2
0
8
6
4
2
0
Chase
Nudge
Lead
Lateral
Quiver


F2
CTLM/CTLF
CTLM/CBZF
CBZM/CTLF
CBZM/CBZF
Frequency of chase, nudge and lead
Frequency of lateral and quiver
225
150
75
2
0
8
6
4
2
0
Chase
Nudge
Lead
Lateral
Quiver

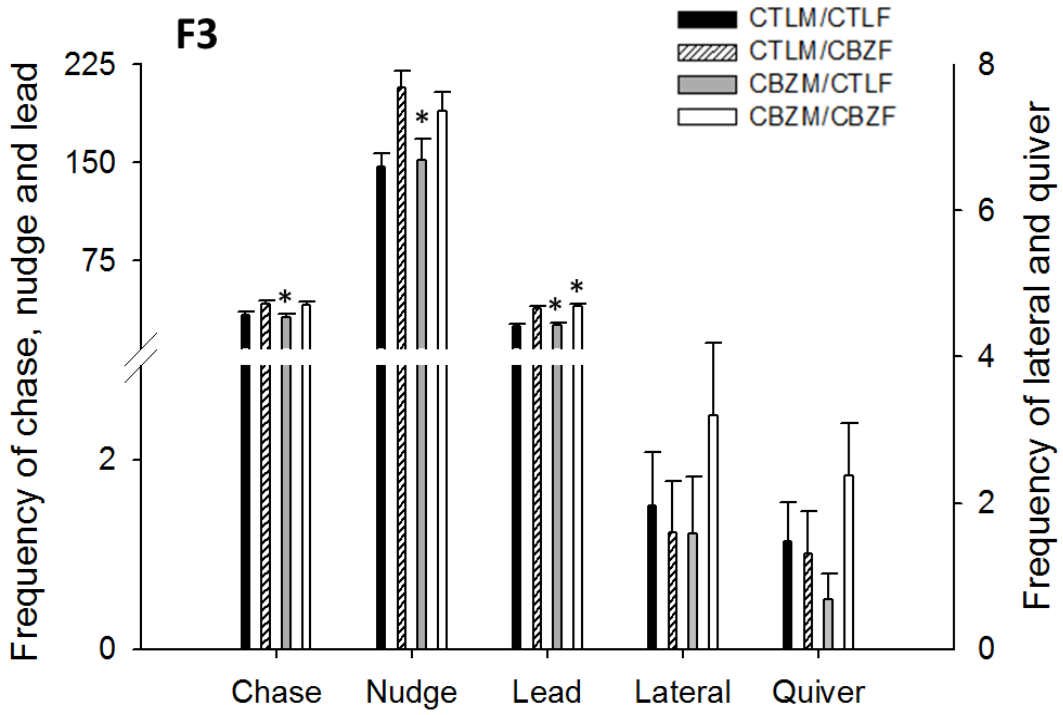
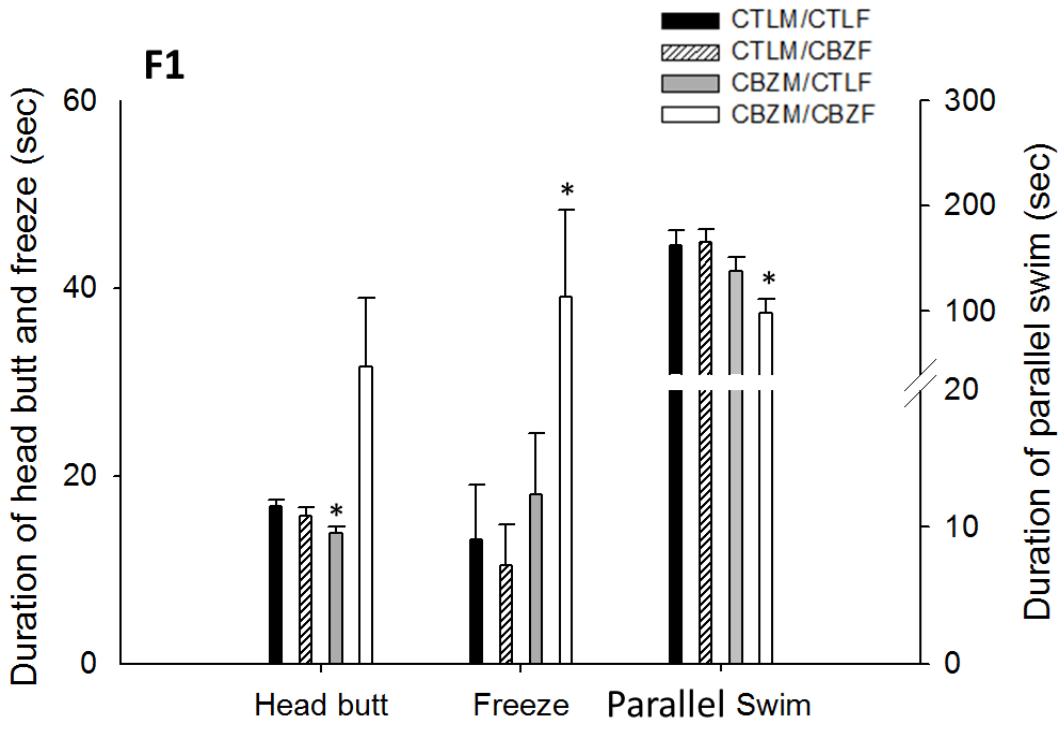
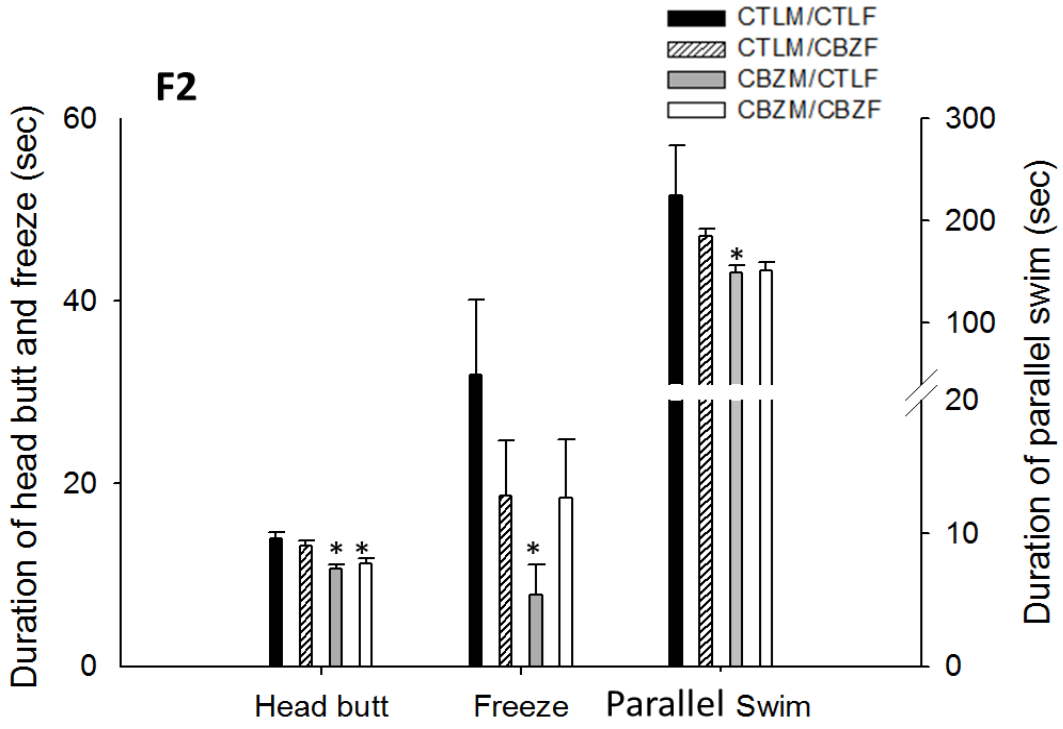


Figure 2.8: Frequencies/counts (mean ± SEM) of male courtship displays in unexposed zebrafish offspring from four lineages. Panels show data from F_1, F_2 and F_3 generations respectively. The figure legend indicates lineages generated with F_0 males (M) and females (F) from unexposed (CTL) and carbamazepine (CBZ) exposure treatments (see section 2.2.3). Courtship videos were taken after first light, when pairs of male and female from the same lineage were crossed together. Videos were scored blind to determine the frequency of five behaviours (see materials and methods for descriptions). Asterisks indicate significant differences from the respective controls, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (nF_1 = 30–42, nF_2 = 34–35, nF_3 = 39–41 videos per group).


F1
CTLM/CTLF
CTLM/CBZF
CBZM/CTLF
CBZM/CBZF
Duration of head butt and freeze (sec)
Duration of parallel swim (sec)
0
20
40
60
0
10
20
100
200
300
*
*
*
Head butt
Freeze
Parallel Swim


F2
CTLM/CTLF
CTLM/CBZF
CBZM/CTLF
CBZM/CBZF
Duration of head butt and freeze (sec)
Duration of parallel swim (sec)
0
20
40
60
0
10
20
100
200
300
*
*
*
*
Head butt
Freeze
Parallel Swim

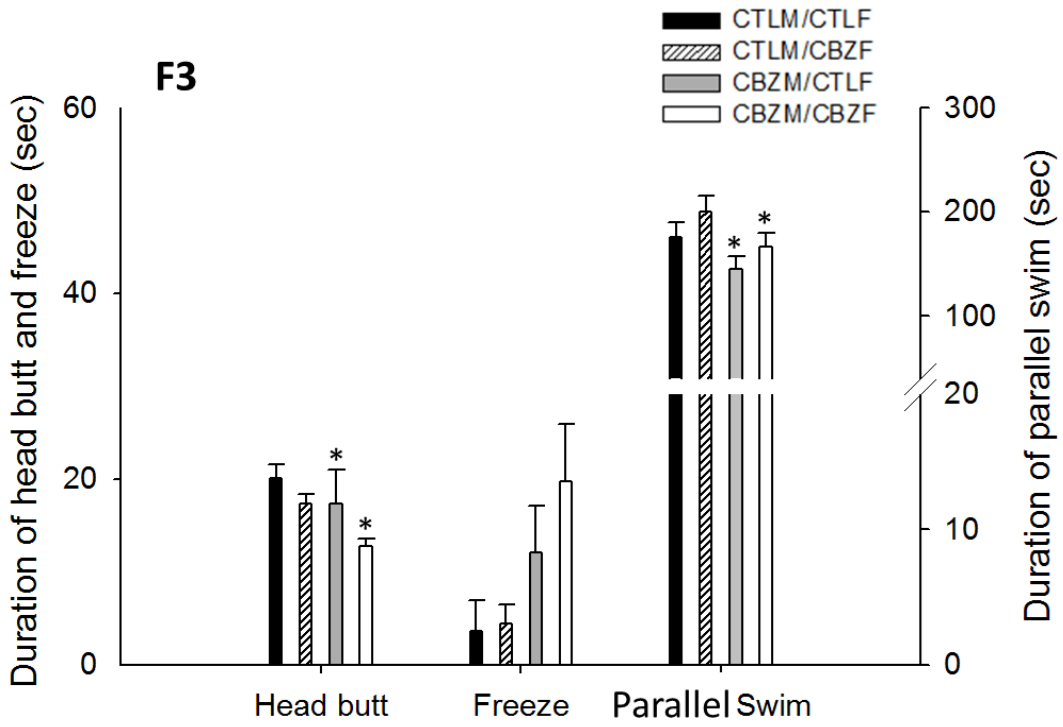


Figure 2.9: Time duration (mean ± SEM) of male aggressive displays in unexposed zebrafish offspring from four lineages. Panels show data from F_1, F_2 and F_3 generations respectively. The figure legend indicates lineages generated with F_0 males (M) and females (F) from unexposed (CTL) and carbamazepine (CBZ) exposure treatments (see section 2.2.3). Videos of mirror interactions were taken from male fish belonging to the four lineages. The videos were scored blind to determine duration of three behaviours (see materials and methods for descriptions). Asterisks indicate significant differences from the controls, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (nF_1 = 28–41, nF_2 = 37–39, nF_3 = 38–42 videos per group).

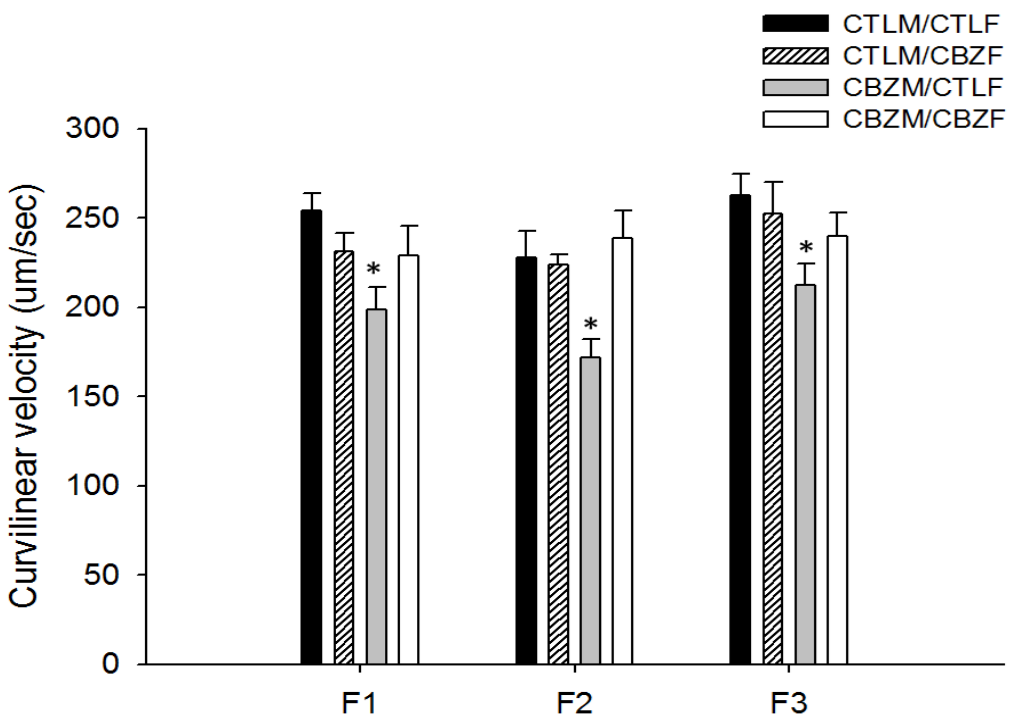


Figure 2.10: Curvilinear velocity (VCL, mean ± SEM) of zebrafish sperm at 20 sec post activation in unexposed male offspring from four lineages of F_1, F_2 and F_3 generations. The figure legend indicates lineages generated with F_0 males (M) and females (F) from unexposed (CTL) and carbamazepine (CBZ) exposure treatments (see section 2.2.3). Milt samples were collected from male fish, activated with system water, and videotaped using Astro IIDC recording software and Olympus CX41 light microscope mounted with Prosilica EC-650 digital camera at 200x magnification, with subsequent analysis of edited video clips to assess sperm velocity through Ceros computer assisted sperm analysis. Asterisks indicate differences from controls, Linear model; $p \leq 0.05$, (n = 22 fish per group).

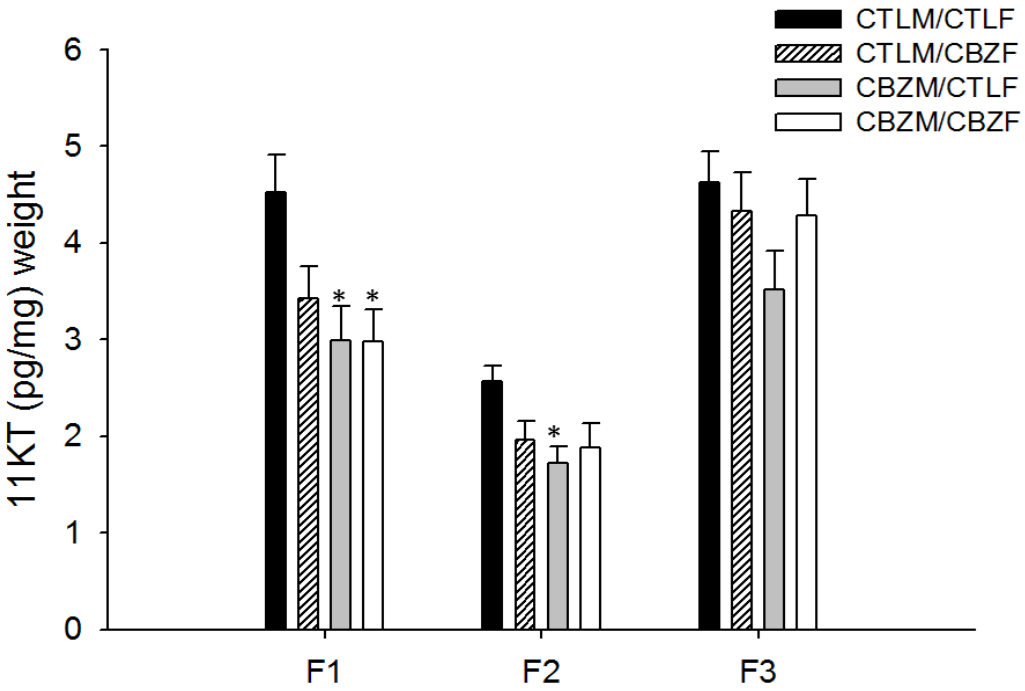
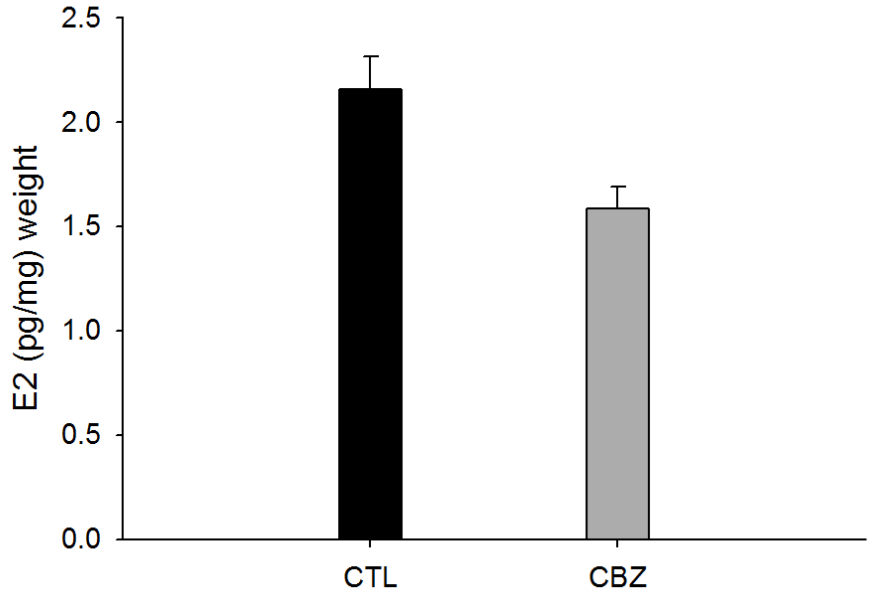


Figure 2.11: Levels of 11-Ketotestosterone (mean ± SEM) in whole body homogenates of unexposed male offspring from four lineages of F_1, F_2 and F_3 generations. The figure legend indicates lineages generated with F_0 males (M) and females (F) from unexposed (CTL) and carbamazepine (CBZ) exposure treatments (see section 2.2.3). Asterisks indicate significant differences from the controls, ANOVA with Holm-Sidak test, $p \leq 0.05$ ($nF_1 = 15–18$, $nF_2 = 14–15$, $nF_3 = 20$).



Suppl. figure 2.1: Levels of estradiol (mean ± SEM) measured in whole body of F_0 females exposed to 10 µg/L of carbamazepine (CBZ), CTL is unexposed animals. ANOVA with Holm-Sidak test, $p \leq 0.05$, (nCTL = 15, nCBZ = 18).

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CHAPTER 3

PARENTAL GEMFIBROZIL EXPOSURE IMPACTS ZEBRAFISH F_1 OFFSPRING, BUT NOT SUBSEQUENT GENERATIONS

Shamaila Fraz, Abigail H. Lee, Adam Khalaf, Krishna Srinivasan, Abhilasha Vermani, Ephraim David, and Joanna Y. Wilson

ABSTRACT

Gemfibrozil (GEM) is a fibrate lipid regulator and one of the most commonly occurring fresh water pharmaceuticals. The negative effects of fibrates including GEM on fish reproduction have been frequently reported and previous research demonstrated negative effects of F_0 GEM exposure on reproduction of the unexposed F_1 offspring. We predicted that chronic, direct exposure of zebrafish with low concentrations of GEM may adversely affect parental male reproduction and unexposed offspring for multiple generations. Adult zebrafish were exposed to 10 µg/L GEM for 6 weeks and a range of reproductive indices were analyzed. The F_1, F_2 and F_3 offspring were reared in clean water from 3 distinct lineages where only a single or both parents were exposed and compared to a control lineage where parents were unexposed. Reproductive indices were examined in unexposed F_1 to F_3 offspring to test the hypothesis of multi-generational impacts. Exposure to GEM caused a decline in breeding success and mean embryo production in F_0 parents and a reduction in whole body 11-ketotestosterone (11KT), altered male courtship, aggression and sperm morphology. Our results indicate that paternal exposure alone is sufficient to result in reproductive effects in unexposed male

offspring. We suggest that GEM may act as reproductive endocrine disruptor in fish and that chronic exposure reduced male reproductive fitness.

3.1 INTRODUCTION

In the last two decades, there have been increasing investigations of the risk to wild fish from exposure to human and veterinary pharmaceuticals (Daughton & Ternes, 1999; Fent et al., 2006; Crane et al., 2006; Overtuf et al., 2015; Corcoran et al., 2010). This is mainly driven by the high frequency of detection in surface water around the globe, the inherent bioactivity of pharmaceuticals and pseudo persistence due to continual release and insufficient removal through waste water treatment (Gavrilescu et al., 2015; Sanderson et al., 2004; Santos et al., 2010; Fent et al., 2006). Hence concerns are brought up about potential adverse effects in fish due to chronic or lifelong exposures (Fent et al., 2006; Daughton & Ternes, 1999; Hughes et al., 2013).

In surface waters, the ubiquity of gemfibrozil (GEM, a lipid regulator) is widely reported (Zenker et al., 2014; Gavrilescu et al., 2015; Verenitch et al., 2006; Kostich et al., 2014; Lishman et al., 2006). Its presence correlates with high prescription rates and sales volume in industrialized societies (Corcoran et al., 2010; Fent et al., 2006). Less than 5% of parent compound is excreted in human urine (Zimetbaum et al., 1991), suggesting that GEM metabolites may also be significant contaminants in wastewater effluent. Being acidic in nature, GEM remained in the aqueous phase with little tendency to partition to suspended biosolids (Araujo et al., 2011) and the maximal removal rates during waste water treatment varied between 10 – 75% (Fent et al., 2006). GEM is

resistant to photo degradation and has an experimental half-life between 119 – 288.8 days in water (Araujo et al., 2011).

In Europe and North America, the maximum levels of GEM in treated municipal waste water were 4760 ng/L and 2300 ng/L respectively (Andreozzi et al., 2003; Koné et al., 2013; Metcalfe et al., 2003; Lishman et al., 2006; Kostich et al., 2014). The maximum surface water concentrations were 7780 ng/Lin Spain (Muñoz et al., 2009), 790 ng/L in USA (Kolpin et al., 2002), and 580 ng/L in Canada (Koné et al., 2013). In a nationwide US survey, GEM was detected in wild caught sunfish (Ramirez et al., 2009). A plasma BCF of 113 was reported in goldfish exposed to 1.5 µg/L for 14 days (Mimmeault et al., 2005), and 63 in rainbow trout acutely exposed to 15 µg/L (Brown et al., 2007).

GEM belongs to the fibrate class of lipid regulators and is a known peroxisome proliferator activated receptor alpha (PPARα) ligand in humans. It increases plasma HDL cholesterol, expression of lipoprotein lipase and Acyl-COA synthetase, and decreases plasma triglycerides and LDL cholesterol (Chinettei et al., 2000; Saku et al., 1985). Based on mammalian data, the effects of fibrates on lipid homeostasis in fish were examined in several studies. Fibrates produced modest peroxisomal responses in rainbow trout, medaka (Scarano et al., 1994), goldfish (Mimmeault et al., 2006), and grass carp (Du et al., 2008). Overall hypolipidemic effects were detected in rainbow trout, (Prindiville et al., 2011; Du et al., 2004), grass carp (Du et al., 2008), fathead minnows (Skolness et al., 2012), and zebrafish (Al-Habsi et al., 2016; Velasco-Santamaría et al., 2011).

Male derived sexual endocrine disrupting effects of fibrates in fish due to chronic exposure have been previously reported. Clofibrate and gemfibrozil decreased plasma

testosterone in male fathead minnows and goldfish, exposed through water (Mimmeault et al., 2005; Runnals et al., 2007). Water borne clofibrate exposure reduced sperm counts, number of viable sperm and plasma 11KT in male fathead minnows (Runnals et al., 2007). Moreover, dietary exposure to bezafibrate decreased plasma testosterone, altered expression of steroidogenic genes, and spermatogenesis (Velasco-Santamaría et al., 2011); and food borne exposure to clofibrate suppressed zebrafish fecundity (Coimbra et al., 2015).

Six weeks water borne exposure of adult zebrafish to 0.5 and 10 µg/L GEM led to a suppression of fecundity (Galus et al., 2013; 2014). Histological scores of the testes were not different from the controls, and no impacts on spermatogenesis were identified (Galus et al., 2013). However, a 10 µg/L exposure reduced the quality of eggs and vitellogenin content in female ovaries, with an increased incidence of atresia at a 0.5 µg/L exposure (Galus et al., 2013). Offspring from parental fish exposed to 10 µg/L GEM were reared in clean water and fecundity, breeding success and male courtship were reduced in crosses between GEM exposed males and control females (Galus et al., 2014). Sperm velocity and morphology in F_1 offspring from GEM exposed fathers were different from controls (Galus et al., 2014).

The aim of the current study was to examine the effects of chronic parental exposure to GEM in unexposed offspring across multiple generations. Offspring effects after parental exposure was considered important because endocrine disruption can cause delayed toxicity and effects may persist in offspring due to parental exposure alone (reviewed by Schwindt, 2015). Hence, the objectives of current study were to analyze the

following questions. Does chronic parental exposure to GEM impact unexposed offspring over F_1–F_3 generations? Does the maternal or paternal exposure history impact the offspring equally?

3.2 MATERIALS AND METHODS

3.2.1 Fish Care and Housing

A multi rack zebrafish holding system (AHAB, Pentair Aquatic Ecosystems Inc. USA) held adult wild type fish (AQuality, Missisauga, Ontario) at a density of 3 fish/L, 30 fish per tank, at equal sex ratios and 14:10 hour day night cycle. The fish were fed tropical fish flakes (Nutrafin Max tropical fish flakes, Hagen, USA) in the morning and the evening and live adult *Artemia nauplii* (Atremia Cysts, Inve aquaculture nutrition, USA) at noon. Fish holding system water was prepared via automatic dosing of stock solutions of sodium bicarbonate (Aquatic Eco systems Inc. USA) and Instant Ocean sea salts (Instant Ocean salts, Spectrum brand, USA) to distilled water, controlled through an electronic monitor (YSI brand, YSI Inc. USA). The housing system was programmed to renew 10% water every day and maintain optimal water temperature of 27–29.0^{o}C, pH from 6.8–8.0, dissolved oxygen 80–100%, and conductivity at 300–400 µS. All methods involving the use and handling of fish were approved by the McMaster Animal Ethics Review Board under the Animal Utilization Protocol # 16-09-34 to JYW.

3.2.2 F_0 Exposure

Before the exposure experiment started, reproductive output of the fish was assessed daily over 14 days by placing embryo traps in each tank before the first light and

collecting embryos about 1.5 hour later. Tanks with comparable reproductive output (mean embryo production per female) were selected, randomly assigned to a replicate tank of either control or GEM treatment, and fish were relocated into experimental glass aquaria with reciculating water. Fish were habituated to tanks for 48 hours prior to initiating exposures. Stocks and dosing solutions of GEM (Sigma Aldrich, Canada) were prepared using DMSO (Caledon Lab Chemicals, USA) and stored at -20^{o}C as single use aliquots. The final concentration of DMSO was 0.004% in all the tanks. There were triplicate tanks in each of the two treatment groups i.e. solvent CTL and 10 µg/L of GEM. A dose renewal with 90% water change out was implemented every third day, and weekly checks for conductivity, nitrites, nitrates, ammonia, dissolved oxygen, and general and carbonate hardness (handheld YSI, YSI incorporated USA; Nutrafin Test, Hagen, USA) were conducted throughout the exposure. An outline of F_0 exposure and endpoint sampling is given in fig. 3.1A.

3.2.3 Breeding of F_0 to Compose Four Offspring Lineages

After six weeks of exposure, and daily monitoring of reproduction by whole tank mating, males and females were mated in pairs, to establish four cross combinations and respective offspring lineages. There were four breeding combinations. Two combinations had pairs of control males and females (CTLM/CTLF) or exposed males and females (GEMM/GEMF). The other two combinations had control males bred with exposed females (CTLM/GEMF) or exposed males mated with control females (GEMM/CTLF). Breeding success was determined by counting the number of successful spawns (out of a total of 75 crosses), and number of embryos per spawn were counted to determine mean

embryo production per female. There were 75 crosses in each group and a single pair was crossed once only. Beacuse of feasibility contraints, pairwise breeding assessment was completed in 17 days, arranging 32 crosses per day. Mating pairs were set up overnight and returned to the exposure tanks the next morning. Different mating combinations were slected randomly on each day to ensure that crosses were paired from each tank and treatment for the whole period of 17 days.

The fish were given 1.5 hour after first light to breed. Embryos were counted under a microscope, rinsed, and plated in 120 x 20 mm glass petri dishes at a density of 150 per dish, in E3 embryo medium (NaCl–292 mg/L, KCl–2.83 mg/L, $MgSO_4$–5.5 mg/L, $CaCl_2$–5.5 mg/L) at 28°C. Viable embryos were determined by dividing embryos at 6 hours post fertilization (hpf); non viable embryos were dead at this time point. From 1 to 5 day post fertilization (dpf), embryos were reared at 28°C and a density of 250 in rectangular food grade plypropylene boxes (19.5 x 11.5 x 8.5cm) holding 250–300 ml of clean E3 embryo medium. Water quality was maintained thorough frequent cleaning and subsequent removal of dead embryos. Equal w/w mixture of Grade 0 food (Hatch fry encapsulation Grade 0, Argent chemical labs, USA) and finely crushed fish flakes (4 times daily) was started from 5 dpf. *Artemia nauplii* larvae were provided to hatchlings from 8 dpf onwards with every feed. Water quality was maintained through 90% daily renewal of E3 medium once daily. Growing hatchlings were gradually transitioned from E3 medium to system water from 12–16 dpf, habituated to a gradual increase in water column height and powder food of bigger particle size by replacing Grade 0 with Grade 1 at 21 dpf, and Grade 1 by Grade 3 at 30 dpf (Hatch fry encapsulation Grade 0, Grade 1 &

Grade 3, Argent chemical labs, USA). Finally, at 30 dpf, the hatchlings were relocated to the multi rack system at a density of 50 fish per 3 liter and maintained at a flow rate of 75–80 ml/minute. From 60 dpf, the feeding regimen constituted 3 feeds per day (powder twice and adult *Artemia* once) and fish were maintained at 3 fish/liter density. The offspring from the four lineages of the subsequent generations were reared under same conditions. An outline of the time line of experiments and endpoint sampling from the F_1–F_3 generations are shown in fig. 3.1B.

3.2.4 Behavioural Assays

Six weeks after the continuous water borne exposure, aggressive behaviour in CTL and GEM exposed males was videotaped in pairwise breeding tanks (Pentair Aquatic Ecosystems Inc. USA); a small tank (~ 700ml of water) suitable for holding one male and female, separated by a removable divider, and slits at the bottom for egg collection. One male was shifted to clean system water in the breeding tank and provided 10 minutes for habituation. After habituation, a mirror was quickly inserted along the side of one wall and the interaction was videotaped for 5 minutes with a Cannon Legria HF R56 camera. The tank was held in a water bath at 28^{o}C. All the videos were played in VLC media player at slow speed and scored blindly to determine frequencies (counts) and the time durations of three displays. Head butting/biting the mirror image, and swimming parallel to the mirror image were used to determine aggression and freezing was scored as an index of anxiety/fear/stress (Kalueff et al., 2013). The sample sizes were 80–126 in F_0, 37–41 in F_1, 31–39 in F_2 and 30–41 in F_3 generations, respectively.

After recording the mirror test, one female was added to the tank, but separated from the male by a divider. The breeding tanks were held overnight at 28^{o}C and courtship behaviour was videotaped for 10 minutes, immediately at first light the next morning, after removing the dividers. All the videos were recorded in the peak spawning window of 1.5 hours after first light. Previously recognized male displays like Chase, Nudge, Lead, Lateral, and Quiver (Darrow & Harris, 2004) were quantified for frequency and duration, through playbacks of videos at slow speed by a blind assessor (nF_0 = 35–42, nF_1 = 38–42, nF_2 = 33–35, nF_3 = 40 videos per group). Chase is defined by the male quickly following the female while nudge is the recurrent touching on female belly or tail. Lead describes the male swimming in tight circles, figure 8 patterns or to the same point more than 3 times. Lateral signals the alignment of genital openings; and quiver represents the male vibrating his body at high frequency and low amplitude to stimulate oviposition in the female, which integrates the release of sperm and eggs at same time (Darrow & Harris, 2004; Kalueff et al., 2013; Spence et al., 2007). The videos where the fish behaved sedentarily for more than 75% of the total time of recording were excluded from the statistical analysis, in both behavioural assays (male aggression and courtship).

3.2.5 Sperm Quality Assessment

Males were allowed to accumulate milt by isolating from females overnight before sampling. Fish were anesthetized with 0.006 mM MS-222 (Sigma Aldrich, Canada) solution buffered at pH 7, and positioned in a wet sponge. Milt was ejected through gentle massage on lateral sides of the fish, in a pre-weighed capillary tube attached with a rubber aspirator. The weight of the sample was used to estimate milt volume under the

assumption of a density equal to water (Dulka et al., 1987). The sample sizes were 34–47 in F_0, 41–48 in F_1, 42–45 in F_2 and 25–38 in F_3 generations, respectively.

Milt samples were fixed in 50 µl of neutral buffer formalin (Fisher, Canada) for 1–2 hour and 20–25 sperm per individual sample were photographed using a Motic BA310 microscope at 400x magnification (n = 25 fish per group and 20 sperm per fish). The sperm head length and width, mid-piece length were measured by drawing a straight line; and tail length by freehand line applying National Institutes of Health's ImageJ software (available at http://rsb.info.nih.gov/ij/).

Milt samples from a second set of fish were diluted with 50 µl of system water at 28^{o}C, mixed quickly, and sperm swimming speed was recorded for 60–70 seconds using Astro IIDC recording software with a sperm tracker system, an Olympus CX41 light microscope mounted with Prosilica EC-650 digital camera at 200x magnification. To determine sperm speed with Ceros CASA (computer assisted sperm analysis), each video was edited to clips of 1 second each, at every 10 sec interval from the beginning using iMovie, (20, 30, 40 and 50 sec). VAP-the velocity on a computed smooth path and VCL-the actual velocity along the swimming track (Rurangwa et al., 2004) were analyzed statistically (n = 22 fish per group).

3.2.6 Steroids Assay

Males were sampled, euthanized, weighed and frozen in liquid nitrogen for whole body 11KT measurements (nF_0 = 15–18, nF_1, nF_2 = 15 nF_3 = 20 fish per treatment group). Whole body estradiol was measured in directly exposed F_0 females only (n = 15–18). The

extractions were performed by method described by Alsop & Vijayan, 2008. Five times w/v of water was added to the frozen whole fish and homogenized (Omni International, GLH tissue homogenizer) for 60 seconds. A portion of homogenate was extracted with diethyl ether repeatedly 3 times. The extracts were combined, evaporated to dryness in a water bath at 37^{o}C, followed by air drying for 2 hours and resuspension in ELISA buffer (Cayman Chemical, Ann Arbor, MI, USA). The suspensions were kept at 4^{o}C with periodic vortexing during 12 hours and finally stored at -80^{o}C for further use. 11KT was measured in homogenates from males and estradiol was assayed in females. Steroids were analyzed in duplicate wells using a colorimetric 96-well EIA kit (Cayman Chemical, Ann Arbor, MI, USA). The extraction efficiency was between 76–80%. The experimentally determined limit of detection for the 11KT assay was 1.37 ± 0.23 pg/ml (mean ± S.D), and inter and intra-assay coefficients of variation were less than 18% and 5% respectively. The experimentally determined cross reactivity of 11KT ELISA kit for testosterone was 0.016 %. The experimental limit of detection for estradiol assay in females was 18.44 ± 2.35 pg/ml (mean ± S.D) and inter- and intra-assay coefficients of variation were less than 14% and 4% respectively.

3.2.7 Statistics

Pairwise breeding (F_0–F_3) was determined as mean embryo production per female, at the end of expsoure, and analyzed by Mann Whitney rank sum test with Bonferroni correction. Breeding success was analyzed by Chi square test with Bonferroni correction. The behavioural (courtship behaviour and aggression) and sperm morphometry results (F_0–F_3) were analyzed by Kruskal-Wallis ANOVA with Dunn's test. Hormonal data

(11KT and estradiol) were analyzed by ANOVA with Holm-Sidak test. A linear regression model was used to interpret data of sperm swimming velocities; the Q–Q Plots were analyzed to confirm the assumptions of residues with normal behaviour graphically and the confidence interval was set at 95%. Differences in F_0 were assessed through multiple comparisons with controls (CBZ vs CTL), with control breeding pair (CTLM/CTLF), or with the control lineage in F_1–F_3 generations. All data analysis was done using Sigma Plot version 10.0, except sperm speed which was analyzed using R version 3.2.2. All data are described as mean ± SEM wherever relevant except sperm morphometrics (medians are reported in Table 3.3); and the level of statistical significance was fixed at $p \leq 0.05$, unless readjusted through Bonferroni correction $p \leq 0.01$.

3.3 RESULTS

3.3.1 Effects of Direct Exposure of F_0

Mean embryo production per female, determined via pairwise breeding (Fig. 3.2A), was significantly lower in crosses consisting of both parents exposed (GEMM/GEMF). Breeding success was decreased in the same crosses (GEMM/GEMF) since pairs successfully spawned only 13 times, whereas the control fish (CTLM/CTLF) spawned 32 times out of a total of 75 crosses (Fig. 3.2B). There were no statistically significant differences in unfertilized and non-viable eggs and the survival of embryos/hatchlings across treatment groups (data not shown).

The most pronounced effects on courtship behaviour were observed in all the crosses with GEM exposed males (Fig. 3.3). The males in the two lineages with GEM exposed fathers (GEMM/GEMF and GEMM/CTLF) had shorter durations of chase, nudge, and lateral displays. The durations of chase were reduced in the CTLM/GEMF cross. Exposure to GEM resulted in shorter durations of freeze behaviour in front of a mirror as compared to the control males. However, the parallel swim and head butt/biting displays were not different relative to the control males (Fig. 3.4).

Milt volume and sperm speed were not found to vary between the GEM exposed and control males (data not shown). However, exposed males had sperm with bigger tails, heads and mid piece (Fig. 3.5). The levels of 11KT in whole body were found to be significantly lower in GEM exposed males relative to the controls (Fig. 3.6) There were no statistical differences in whole body estradiol levels in exposed versus control females (Suppl. Fig. 3.1).

3.3.2 Effects in Offspring

Mean embryo production in F_1–F_3 generations were determined by pairwise crossing of males and females within each lineage (Fig. 3.7). Embryo production was highly variable and no differences were detected amongst the lineages in the F_1 and the F_2 offspring; however, the F_3 offspring from GEMM/CTLF lineage had significantly (54.3%) lower reproductive output than the controls. This lineage had the lowest embryo production in each generation. The unfertilized and non-viable eggs and the survival of

embryos/hatchlings showed no statistically significant differences relative to the control lineage in any generation (data not shown).

Male courtship towards a female of the same lineage was scored in F_1–F_3 generations; the durations of five courtship displays are presented in Fig. 3.8 and the frequencies (counts) are summarized in Table 3.1. The F_1 males from the GEMM/CTLF and GEMM/GEMF lineage spent less time performing lead and quiver (Fig. 3.8A) as compared to F_1 CTLM/CTLF males. The F_1 males from GEMM/CTLF had reduced durations of chase and CTLM/GEMF lineage had reduced durations of chase, lead and quiver (Fig. 3.8A). Courtship behaviour in F_2 and F_3 males from all lineages was similar (Fig. 3.8B).

The time durations of aggressive displays are presented in Fig. 3.9 and the frequencies (counts) are summarized in Table 3.2. The F_1 males from CTLM/GEMF lineage had shorter time durations of parallel swims to the mirror as compared to control offspring (Fig. 3.9A). The F_2 male offspring from the lineage GEMM/GEMF had shorter durations of freezes in front of the mirror (Fig. 3.9B). No significant differences in interaction with mirror were found in F_3 male offspring (Fig. 3.9C).

No changes were found in milt volume (data not shown). Morphological changes in sperm were found in the F_1–F_3 offspring of the GEMM/CTLF and GEMM/GEMF lineages only (Table 3.3). For GEMM/CTLF lineage, head diameter was smaller in F_1 and F_3 sperm but larger in F_2 sperm. Head lengths were larger than the control sperm in F_1 and F_2 offspring but smaller in F_3 offspring. The sperm mid piece length was shorter in

both F_1 and F_2 fish and tails were longer in both F_1 and F_3. GEMM/GEMF lineages had smaller head length in F_1, larger head diameter and head length in F_2, and smaller head length in F_3 generations. Sperm swimming velocity determined by curvilinear velocity (VCL) was greater than the control sperm for the F_2 offspring of the lineage GEMM/CTLF (Fig. 3.10A). Similar differences were found in angular path velocity (VAP, data not shown). Whole body 11KT levels were lower, compared to the control lineage, for F_1, and F_2, but not in F_3 males from GEMM/CTLF lineage (Fig 3.11).

3.4 DISCUSSION

We detected significant declines in mean embryo production of chronically exposed zebrafish, by monitoring fecundity via pairwise breeding (Fig. 3.2). The magnitude of fecundity decline in exposed zebrafish quantified by whole tank breeding from day 1–42 of exposure (not shown here), was in close agreement with previous research (Galus et al., 2013; 2014) using the identical dose, exposure method and the dosing regimen for GEM as reported here. In fact, five separate identical exposures of adult zebrafish with GEM (including the work by Galus et al., 2013; 2014, Chapter 4, and J.Y. Wilson, unpublished data) have been replicated in our lab with different people conducting the experiments on different batches of fish. A similar reduction in reproductive output in all replicated experiments allowed the use of tank breeding as a biological indicator of exposures confirmation. All endpoints that were previously altered with GEM exposure (Galus et al., 2013; 2014) were similarly impacted in this experiment, confirming that our dosing was equivalently effective. The exposure has been

validated through detailed time course analysis of aqueous concentrations between 10 min–72 hours, which were found to be close to nominal concentrations (Galus et al., 2013). Our aqueous concentrations in a replica experiment were also confirmed to be fairly close to the nominal concentrations (Chap 4).

This study expanded the range of reproductive testing of zebrafish after chronic exposure to GEM by including male aggression, courtship behaviour and sperm quality in F_0 males, while integrating the endpoints examined previously like fecundity and sex steroids (Galus et al., 2013) to ensure consistency between experiments. The broad range of reproductive indices was applied for assessments in multiple generations of unexposed offspring, as parental GEM exposure effects on courtship and sperm in F_1 male offspring had been documented (Galus et al., 2013; 2014). Reproduction and courtship behaviour was assessed in pairwise crosses in four combinations; by crossing male and female from the same treatment (CTLM/CTLF, GEMM/GEMF); and by crossing an exposed male or female with a control partner (CTLM/GEMF, GEMM/CTLF). Further, we reared these lineages for three generations to determine the impacts of parental GEM exposure on offspring.

3.4.1 Effects of direct exposure of F_0

Adverse exposure effects were identified by a lower mean embryo production per female and breeding success in pairwise crosses including exposed males and females (GEMM/GEMF). There was a decrease, although not statistically significant, in the other cross with exposed males (GEMM/CTLF, Fig. 3.2A, B), suggesting that male exposure

history to GEM impacts fecundity measures. Our observations are similar to earlier reports of declining fecundity in both adult zebrafish and fathead minnows exposed to GEM (Galus et al., 2013; Skolness et al., 2012); and zebrafish exposed to clofibric acid (Coimbra et al., 2015).

Male derived effects on fish reproduction might be expected after GEM exposure, based on impacts on 11KT level, male courtship, and sperm quality. 11KT is the quantitatively predominant, male specific circulating androgen in many teleost fish including zebrafish (Borg, 1994; Tokarz et al., 2015) and is a physiologically important mediator of male reproduction (Scholz & Myer, 2008). Similar to current study, the androgen lowering effects of fibrates in fish including GEM were found by others (Mimeault et al., 2005; Runnals et al., 2007; Velasco-Santamaría et al., 2011; Prindiville et al., 2011; Cameron, 2011). Lower androgens may have direct effects on associated physiological processes like spermatogenesis, sexual behaviour, aggression (Archand-Hoy & Bensen, 1998; Tokarz et al., 2015).

Effective courtship behaviour is a mandatory condition for successful fertilization (Munakata & Kobayashi, 2010). Inefficient male courtship behaviour was apparent because the durations (Fig. 3.3) and frequencies (Table 3.1) of male courtships displays were less in the crosses involving GEM males (GEMM/CTLF, GEMM/CTLF). The range of effects in the two lineages was quite similar (chase, nudge, and lateral – Fig. 3.3, in addition to quiver – Table 3.1). Shorter durations of chase and nudge may indicate impaired pheromonal signaling, as these displays mark the stimulatory beginning of male courtship and repetition of these displays is needed for productive courtship behaviour

(Kalueff et al., 2013; Darrow & Harris, 2004). Thus, these results broadly reflect impaired male courtship, because chase and nudge are positively correlated with efficacious courtship behaviour, lateral announces alignment of genital pores and quiver stimulates egg release (Darrow & Harris, 2004). Shorter time durations of lateral displays may be representative of both reduced male stimulation and female responsiveness. We considered reduced female responsiveness for two reasons; in the current study, the females exposed to GEM chased away the males in two cross combinations (CTLM/GEMF, GEMM/GEMF, Fraz, personal observations); and previous exposures at this concentration increased incidence of atretic, pre vitellogenic oocytes and fewer mature oocytes (Galus et al., 2013). Together these observations support the hypothesis of reduced female readiness because the presence of mature and vitellogenic oocytes is a precondition for successful courtship behaviour (Munakata & Kobayashi, 2010). Moreover, we suggest that alterations in courtship behaviour might be attributed to changes in androgen levels, as 11KT acts in a fortifying manner for manifestation of productive sexual behaviour after the initial physiological stimulus (Munakata & Kobayashi, 2010), and its elevated levels are associated with male derived behaviour in several teleost fish (Kobayashi & Nakanishi, 1999; Páll et al., 2002; Pankhurst et al., 1999; Stacey & Sorensen, 2009). In zebrafish, the glucoronidated conjugates of male sex steroids were shown to behave as the initial physiological stimulus for courtship (Gerlach, 2006) and male reproductive success was linked with higher levels of 11KT (Coe et al., 2008). Additionally, anti-androgens and estrogens, both of which lower androgens,

suppress male courtship behaviour in fish (Bayley et al., 2002; Colman et al., 2009; Bjerselius et al., 2001).

Sperm quality encompasses whole suite of factors including sperm count, morphology, viability, velocity, longevity and integrity of functional components (Snook, 2005; Gerber et al., 2016). We were not able to evaluate competitive fertilization but noted altered sperm quality in the current study; however, we have characterized significant differences in sperm morphology and lower 11KT (Fig. 3.5), but not in sperm swimming speed, after GEM exposure. Male derived adverse effects of fibrates on fish reproductive physiology are documented by other studies. Histological changes in fathead minnows and zebrafish testes were produced by bezafibrate and clofibric acid exposures (Velasco-Santamaría et al., 2011; Coimbra et al., 2015) but not in zebrafish exposed to GEM (Galus et al., 2013). Reduced sperm count and swimming velocities in adult zebrafish males were noted by clofibric acid exposure (Runnalls et al., 2007). Decreased 11KT was suggested as the mechanism by which clofibric acid impacted spermatogenesis in zebrafish (Runnals et al., 2007), since11KT is necessary for the process of spermatogenesis, and exerts direct hormonal control or indirectly affects paracrine control (Schulz & Miur, 2002). Collectively, these data suggest that fibrates including GEM, impact spermatogenesis and sperm quality.

Negative effects of fibrates on fish reproduction are well recognized but the mechanism is not understood. A notable limiting factor is the poor understanding of function of PPARs and specificity of PPAR agonists in fish, regardless of the fact that various fish including zebrafish express all three PPAR subtypes i.e. PPARα, PPARβ, and

PPARγ (Ruyter et al, 1997; Leaver et al., 1998; Mimeault et al., 2006; Ibabe et al., 2002). There are two ways by which fibrates are implied to affect fish reproduction. Firstly, fibrates may interfere with biosynthesis and breakdown of cholesterol and fatty acids and alter cholesterol availability for production of sex steroids in fish (Velasco-Santamaría et al., 2011), and these effects are well recognized to occur via PPAR activation in mammals (reviewed by Manibusan & Touart, 2017). This hypothesis is supported indirectly by several studies; GEM exposure caused significant reductions in plasma cholesterol and triglycerides in rainbow trout (Prindiville et al., 2011), total cholesterol in male fathead minnows (Skolness et al., 2012), and whole body cholesterol in male zebrafish (Al-habsi et al., 2016). Moreover, a positive correlation between decreased plasma 11KT and cholesterol in male zebrafish was reported with bezafibrate exposure (Velasco-Santamaría et al., 2011). The second possible mechanism for PPAR mediated effects is via direct impacts on gonadal steroid synthesis by altering the steroidogenic signaling pathways, the rate limiting step of cholesterol transport, or via altering transcription of genes, as in rodents (Forment et al., 2006; Gazouli et al., 2002; Liu et al., 1996). Direct effects of GEM and fenofibrate on testicular steroidogenesis were indicated by reduced testosterone production in goldfish testes fragments in vitro (Cameron, 2011).

3.4.2 Effects in the offspring

The parental effects of F_0 exposure to 10 µg/L GEM in unexposed F_1 offspring were observed by Galus et al. (2014). Our study assessed impacts beyond the F_1 generation and suggested paternal effects. Like Galus et al. (2014), we found significant declines in fecundity for GEMM/GEMF (Fig. 3.7), reduced courtship behaviours (Fig.

3.8A, Table 3.1), and changes in sperm morphology in F_1 males from GEM exposed parents (Table 3.3). In both studies, F_1 male offspring had similarly decreased male courtship behaviour, shown by reduced durations of leading display in crosses between F_1 males with GEM parental exposure and control females (GEMM/CTLF) and reduced frequencies of chase and quiver in the crosses between males and females with GEM parental exposure (GEMM/GEMF, Galus et al., 2014). Moreover, smaller mid piece lengths in F_1 males with GEM exposure history were commonly observed by Galus et al. (2014) and the current study.

Galus et al. (2014) reported significantly decreased fecundity in F_1 offspring from GEMM/CTLF lineage, while we observed a decreasing trend (Fig. 3.7). This could be due to the difference in how the pairwise cross combinations were performed between the two studies. We organized pairwise crosses in F_0 to compose specific offspring lineages; while in the former study the F_1 were reared from common parental exposure and the crosses were arranged in the F_1 generation (Galus et al., 2014). We found no significant changes in swimming speed in F_1 males with GEM exposure history, a difference from the former report (Galus et al., 2014). Further, we detected significantly lower 11KT in whole body of F_1 offspring from GEMM/CTLF lineage, an endpoint not measured previously (Figure 3.11). In summary, we largely confirmed male derived effects in the F_1 and but also added to the existing evidence by determining 11KT level and male aggression.

The effects on unexposed offspring were largely limited to the F_1 generation, thus the effects could be regarded as “single generation inter-generational” paternal effects of

chronic exposure, since F_1 were only exposed as germ line (Plaistow et al., 2006). Reports of paternal effects of stressors are frequent in the mammalian literature, but have been limited to EE_2 in fish (reviewed by Aiken & Ozanne, 2014; Curley et al., 2014; reviewed by Schwindt, 2015), in large part because most studies examine the impacts of parental exposure together and do not separate maternal from paternal exposure history. We used multiple breeding combinations, which is a recognized strategy of detecting paternal effects (reviewed by Schwindt, 2015). Nash et al. (2004) showed paternal effects of F_0 EE_2 exposure on fertility, through replacing F_1 males with exposure history by unexposed males. The F_1 male derived effects of F_0 EE_2 exposure were also found in rainbow trout, and were shown to be clearly transmitted though sperm (Schultz et al., 2003; Brown et al., 2007; 2008).

The hypothesis of multigenerational effects of GEM exposure in zebrafish could not be supported because most of the endpoint effects in the unexposed offspring did not persist to F_2 or F_3 generation. Thus, multigenerational effects are likely limited because only F_2 male offspring from a single lineage (GEMM/CTLF) had altered sperm speed and morphology (Fig. 3.10, table 3.3) and lower whole body 11KT relative to the respective controls (Fig. 3.11).

3.5 CONCLUSIONS

The current study showed the "single generation inter-generational" paternal effects of chronic F_0 exposure to gemfibrozil in zebrafish. Although we were not able to detect multigenerational affects, the presence of multiple, negative impacts in unexposed

F_1 offspring (fecundity, sex steroid, male courtship, and sperm quality), indicates that paternal exposure was sufficient to affect reproduction in the unexposed offspring. In addition, we showed that direct chronic 10 µg/L GEM exposure of zebrafish affects male courtship, lowers whole body 11KT in males, and alters sperm morphology; endpoints which had not been previously examined with GEM exposure. Nonetheless, several challenging questions remain. An examination of the roles of fish PPAR subtypes in reproduction, especially PPARα, would be important to determine the underlying mechanism of action and whether this mechanism is similar to mammals. An intriguing aspect for future research is the mechanism of paternal effects and how these effects are transmitted through sperm. Overall, a better mechanistic understanding of male reproductive dysfunction by fibrate exposure in fish would be valuable for risk assessment and predictive toxicology for this frequently occurring class of water contaminants.

3.6 TABLES AND FIGURES

Table 3.1: Frequencies (counts) of male courtship displays in F_0 parents exposed to 10 µg/L gemfibrozil (GEM) for 6 weeks; and unexposed offspring from F_1, F_2 and F_3 generations. Unexposed (CTL) and exposed (GEM) males (M) and females (F) were crossed at the F_0 generation; F_1–F_3 generations were crossed within each lineage. Asterisks indicate significant differences from the respective controls, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (nF_0 = 35–42, nF_1 = 38–42, nF_2 = 33–35, nF_3 = 40 videos per group).

	Description of F_0 crosses OR offspring lineages	Frequencies of courtship displays (Mean ± SEM)				
		Chase	Nudge	Lead	Lateral	Quiver
F_0	CTLM/CTLF	59 ± 5.18	139 ± 15.23	20 ± 2.50	19 ± 20.68	5 ± 1.38
	CTLM/GEMF	46 ± 6.23	89 ± 11.84	21 ± 1.82	8 ± 9.96	1 ± 0.10*
	GEMM/CTLF	26 ± 3.75*	58 ± 11.85*	12 ± 2.21*	5 ± 6.49	1 ± 0.22*
	GEMM/GEMF	24 ± 3.53*	43 ± 5.76*	22 ± 2.85	3 ± 2.75	0 ± 0.18*
F_1	CTLM/CTLF	54 ± 2.78	110 ± 7.24	15 ± 1.06	4 ± 1.04	2 ± 0.80
	CTLM/GEMF	49 ± 5.93	76 ± 9.85*	14 ± 2.01	2 ± 0.77	1 ± 0.86
	GEMM/CTLF	49 ± 4.22	84 ± 9.68*	10 ± 1.45*	2 ± 0.64	1 ± 0.41
	GEMM/GEMF	44 ± 4.00*	89 ± 14.74	9 ± 1.19*	2 ± 1.16	0 ± 0.30*
F_2	CTLM/CTLF	50 ± 4.66	114 ±14.49	18 ±1.57	2 ± 1.37	1 ± 0.41
	CTLM/GEMF	47± 3.48	137 ±16.12	22 ± 2.67	3 ± 1.26	1 ± 0.30
	GEMM/CTLF	43 ± 4.58	136 ± 16.42	33 ± 4.11	2 ± 0.98	0 ± 0.49
	GEMM/GEMF	54 ± 4.01	155 ± 13.78	32 ± 2.11	2 ± 0.70	1 ± 0.21
F_3	CTLM/CTLF	34 ± 1.98	147 ± 10.15	25 ± 1.40	2 ± 0.73	1 ± 0.53
	CTLM/GEMF	47 ± 4.70	178 ± 14.74	43 ± 2.27	2 ± 0.50	1 ± 0.41
	GEMM/CTLF	38 ± 4.34*	158 ± 14.32	26 ± 1.55*	2 ± 0.57	1 ± 0.29
	GEMM/GEMF	39 ± 2.52	182 ± 15.80	31 ± 1.96	2 ± 0.75	2 ± 0.50

Table 3.2: Frequencies (counts) of male offspring aggressive displays in F_0 parents exposed to 10 µg/L GEM for 6 weeks; and unexposed offspring from F_1, F_2 and F_3 generations. Unexposed (CTL) and exposed (GEM) males (M) and females (F) were crossed at the F_0 generation; F_1–F_3 generations were crossed within each lineage. Asterisks indicate significant differences from the respective controls, Kruskal-Wallis ANOVA with Dunn's test; $p \leq 0.05$, (nF_1 = 37–41, nF_2 = 31–39, nF_3 = 30–41 videos per group).

	Description of F_0 males OR offspring lineages	Frequencies of aggressive displays (Mean ± SEM)		
		Head butt	Parallel swim	Freeze
F_0	CTLF	96 ± 3.7	35 ± 1.9	7 ± 0.7
	GEMM	91 ± 4.6	31 ± 2.8	4 ± 0.6
F_1	CTLM/CTLF	84 ± 3.80	9 ± 0.79	1 ± 0.35
	CTLM/GEMF	90 ± 5.59	5 ± 0.62*	1 ± 0.33
	GEMM/CTLF	75 ± 3.61	9 ± 0.68	1 ± 0.36
	GEMM/GEMF	85 ± 3.33	8 ± 0.64	1 ± 0.27
F_2	CTLM/CTLF	75 ± 3.58	6 ± 0.28	2 ± 0.46
	CTLM/GEMF	67 ± 3.74	6 ± 0.40	1 ± 0.37
	GEMM/CTLF	70 ± 2.75	7 ± 0.52	1 ± 0.17
	GEMM/GEMF	83 ± 1.91	5 ± 0.32	0 ± 0.12
F_3	CTLM/CTLF	126 ± 8.93	8 ± 0.87	0 ± 0.08
	CTLM/GEMF	116 ± 8.95	8 ± 0.69	0 ± 0.08
	GEMM/CTLF	128 ± 14.44	8 ± 0.78	0 ± 0.19
	GEMM/GEMF	183 ± 25.97	8 ± 0.63	0 ± 0.08

Table 3.3: Morphometric measurements of sperm in unexposed zebrafish offspring from F_1, F_2 and F_3 generations. Offspring were generated by breeding unexposed (CTL) and exposed (GEM) male (M) and female (F) F_0 zebrafish; $F_1 – F_3$ generations were crossed within each lineage only. For morphological measures, diameter (D) and length (L) were measured for the sperm head, midpiece (MP) and tail. Asterisks indicate significant differences from the controls, Kruskal-Wallis ANOVA with Dunn's test $p \leq 0.05$, (n = 25 fish per group and 20 sperm per sample).

	Parameters of sperm morphology	Sperm Morphometrics μm (Median)				
		CTLM /CTLF	CTLM /GEMF	GEMM /CTLF	GEMM /GEMF	H-Statistic
F_1	Head D.	5.077	5.077	4.714*	4.808	246.80
	Head L.	6.667	6.864	7.196*	6.290*	293.27
	MP. L.	3.336	3.590	3.000*	3.333	102.20
	Tail L.	45.00	46.20	51.60*	46.50	184.20
F_2	Head D.	5.548	5.676	5.907*	5.826*	373.94
	Head L.	6.369	6.369	7.211*	6.852*	384.40
	MP. L.	3.333	3.333	2.828*	3.333	308.34
	Tail L.	52.95	53.01	52.496	54.074	45.09
F_3	Head D.	4.646	4.466	4.164*	4.390	138.53
	Head L.	5.543	5.398	4.524*	5.244*	426.33
	MP. L.	2.142	2.048	2.142	2.020	28.72
	Tail L.	67.23	68.26	70.38*	68.46	202.62

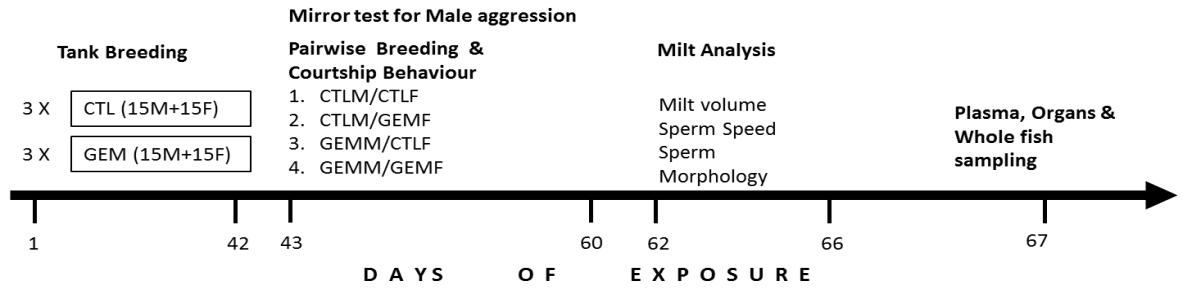


Fig 3.1A: Schematic of direct exposure to 10 µg/L gemfibrozil (GEM) and endpoint sampling in F_0 zebrafish. Exposed and unexposed (CTL) animals were crossed to generate 4 lineages of F_1 offspring.

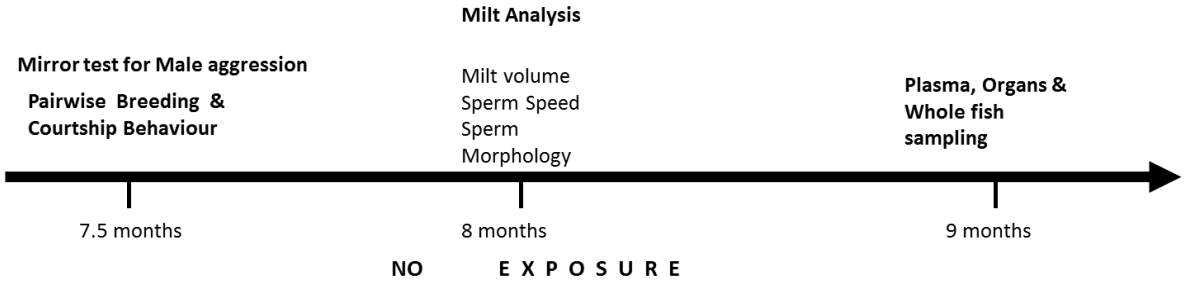


Fig 3.1B: Schematic of endpoint sampling with the unexposed offspring for each of F_1, F_2, and F_3 generations. F_1 – F_3 generations were reared in clean water; the F_0 generation was exposed to control or gemfibrozil treatment.

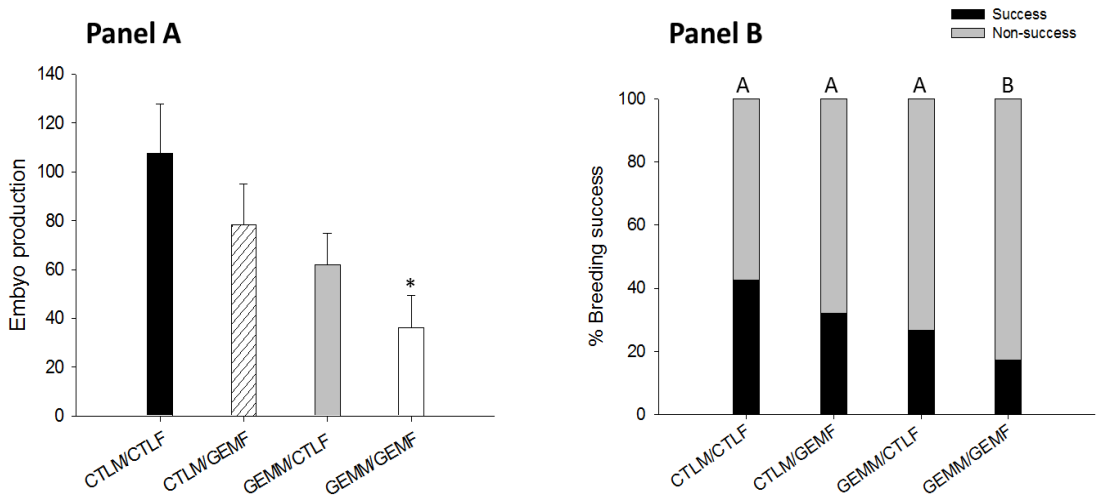


Figure 3.2: Embryo production ((mean ± SEM, panel A) and breeding success (panel B) in four combinations of breeding pairs from F_0 zebrafish after 6 weeks exposure to 10 µg/L gemfibrozil (GEM). X–axes in both panel A and B indicate breeding pair combinations. Control (CTL) males (M) were crossed with either CTL or exposed female (F; CTLM/CTLF and CTLM/GEMF); and GEM exposed males were crossed with either exposed female or CTL female (GEMM/GEMF and GEMM/CTLF). Asterisk and different letters indicate significant differences from the controls. Mann Whitney rank sum test (A) and Chi square test (B) with Bonferroni corrections; $p \leq 0.01$ (n = 75 crosses per group).

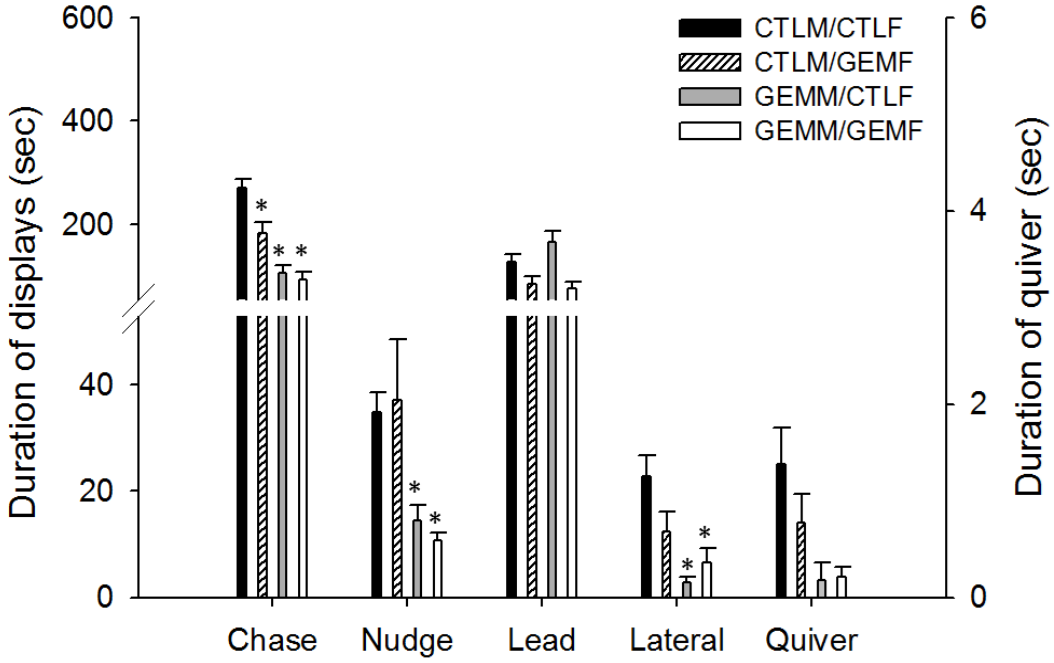


Figure 3.3: Time duration (mean ± SEM) of male courtship displays in F_0 zebrafish after chronic 6 weeks exposure to 10 µg/L gemfibrozil (GEM). Courtship videos were taken after first light in crosses from exposed (GEM) and unexposed (CTL) fish with one male (M) and one female (F) in each tank, to produce the four combinations indicated in the figure legend. Videos were scored blind to determine frequency of five behaviours (see materials and methods for descriptions). Asterisks indicate significant differences from the controls, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (n = 35–42 videos per group).

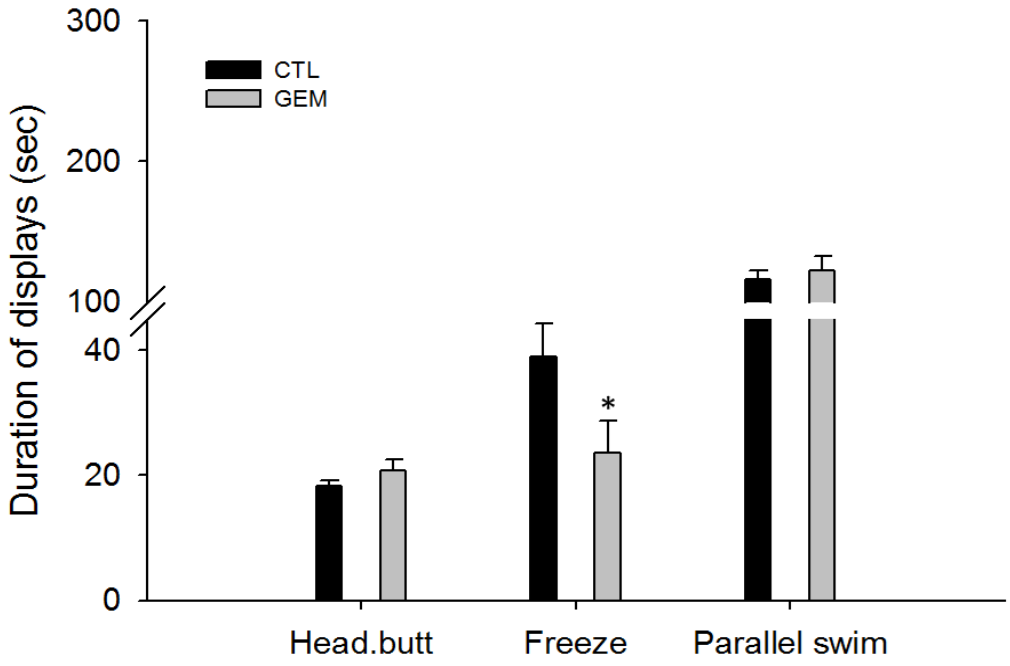


Figure 3.4: Time duration (mean ± SEM) of male aggressive displays (head butt, parallel swims) or anxiety (freeze) in F_0 zebrafish after chronic 6 weeks exposure to 10 µg/L gemfibrozil (GEM). Videos of mirror interactions were taken from exposed (GEM) and unexposed (CTL) male fish. The videos were scored blind to determine duration of three behaviours (see materials and methods for descriptions). Asterisk indicates significant difference from the controls, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (nCTL = 126 and, nGEM = 80).

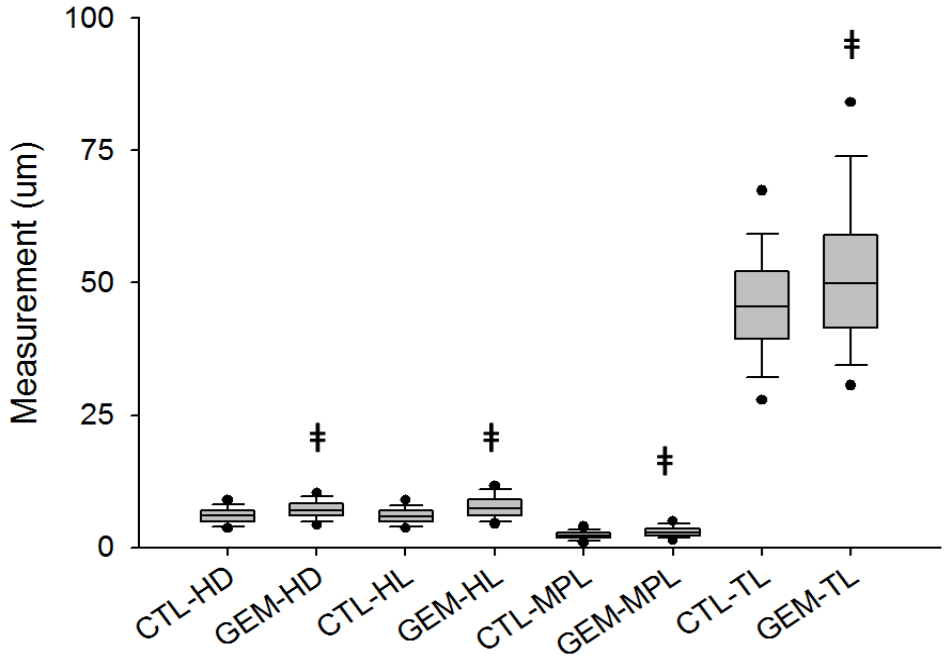


Figure 3.5: Morphometric measurements (mean ± SEM) of zebrafish sperm from F_0 fish exposed to 10 µg/L gemfibrozil (GEM). Milt samples were collected from exposed (GEM) and unexposed (CTL) male fish, fixed in formalin, and 20 sperm per sample were imaged using Motic BA-130 microscope at 400 x magnification. Head diameter (HD), head length (HL), midpiece length (MPL) and tail length (TL) were quantified. Daggers indicate significant differences from the controls, Kruskal-Wallis ANOVA, with Dunn's test, $p \leq 0.05$, (n = 20 fish per group and 25 sperm per sample).

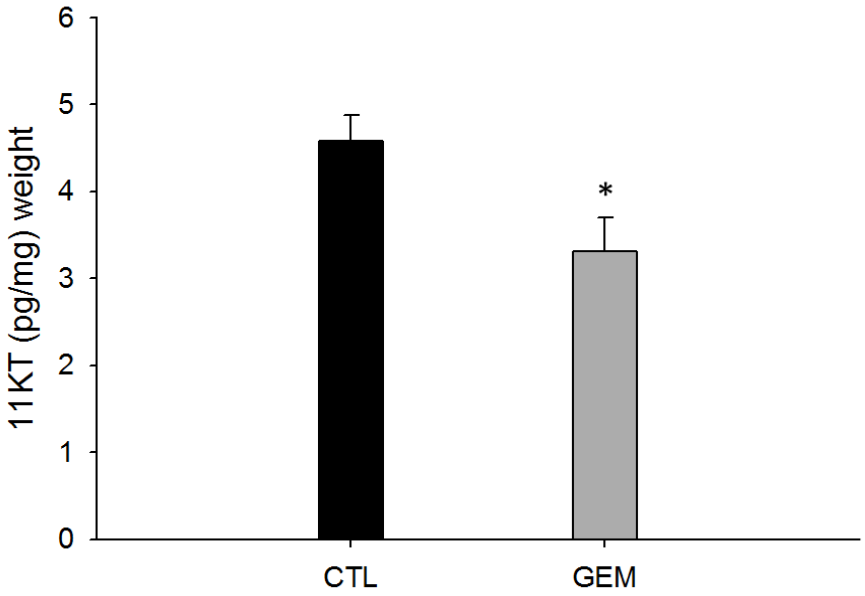


Figure 3.6: 11-Ketotestosterone (mean ± SEM) in whole body homogenates from exposed (GEM) and unexposed (CTL) F_0 zebrafish males. Asterisk indicates significant difference from the control, ANOVA with Holm-Sidak test, $p \leq 0.05$, (nCTL = 15, nGEM = 18).

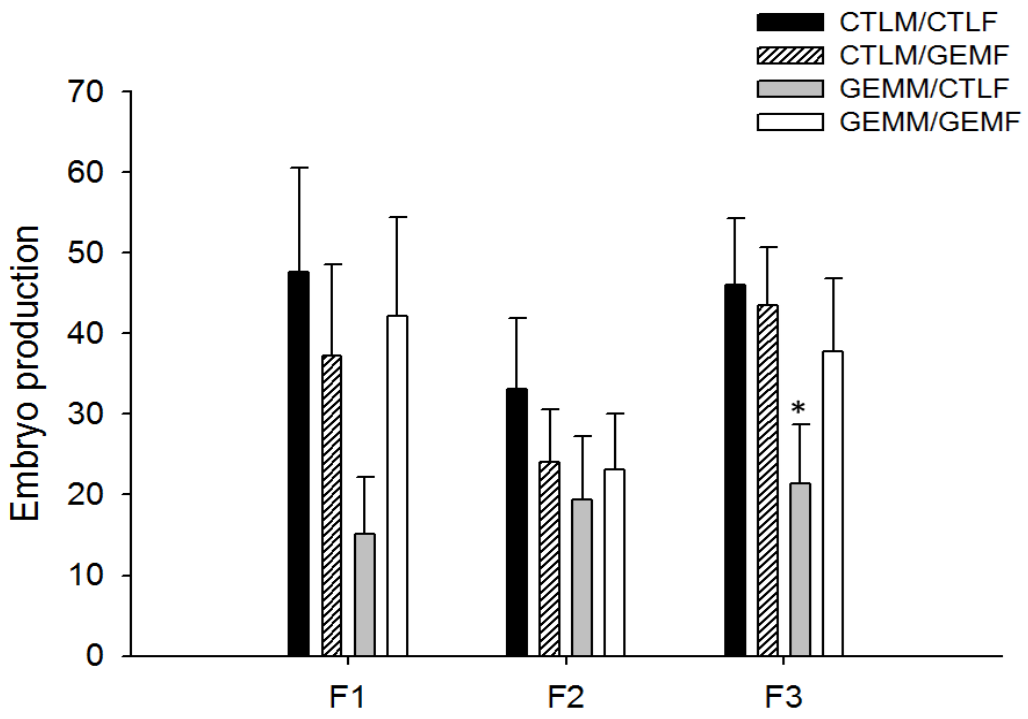
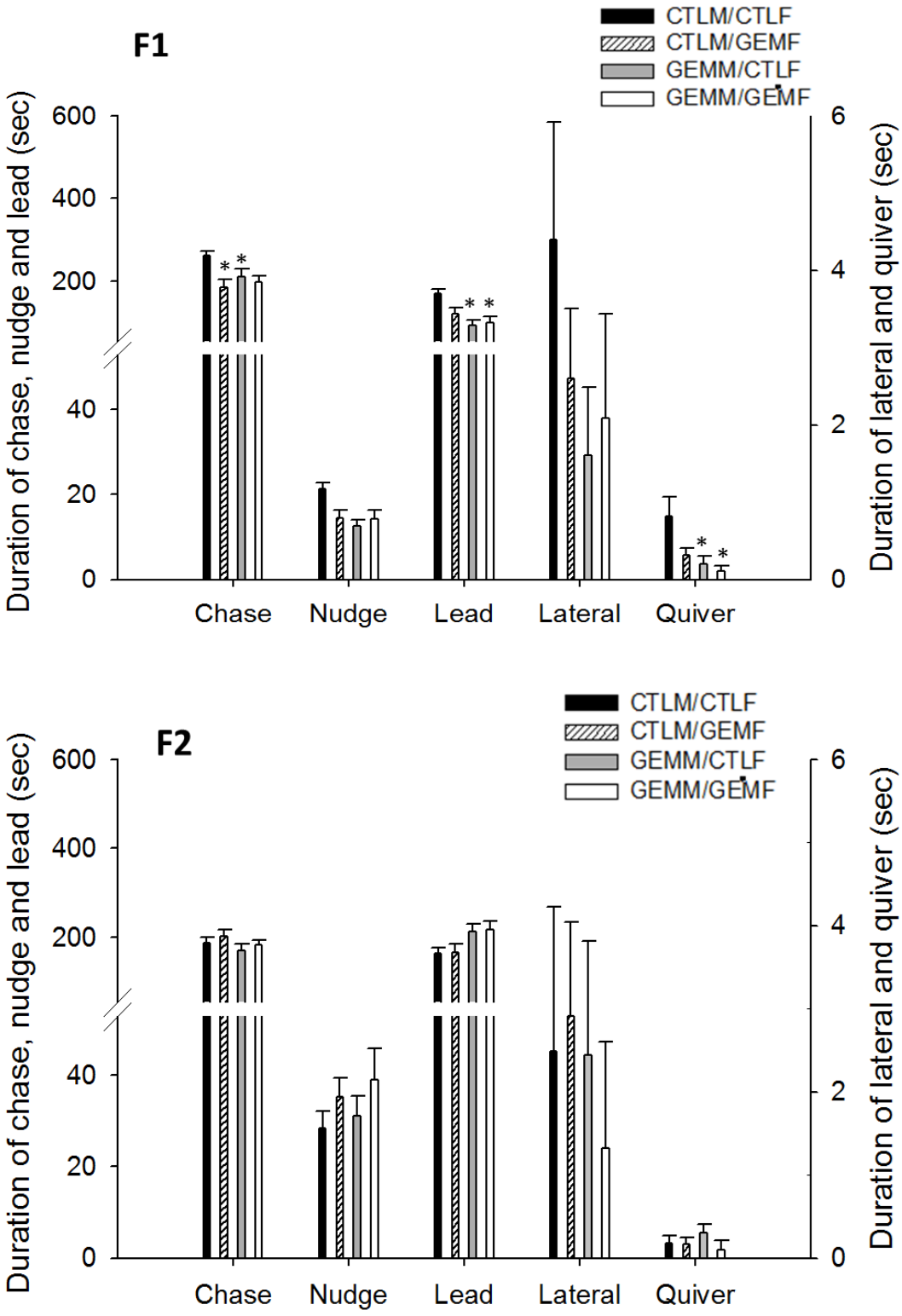


Figure 3.7: Embryo production (mean ± SEM) in breeding pairs of unexposed zebrafish offspring from four lineages over F_1, F_2 and F_3 generations. The figure legend indicates lineages generated with F_0 males (M) and females (F) from unexposed (CTL) and gemfibrozil (GEM) exposure treatments; for F_2 and F_3 generations, pairs of male and female from the same lineage were crossed together (see section 3.2.3). Asterisk indicates significant differences from the respective control lineage within a generation, Mann Whitney rank sum test with Bonferroni corrections; $p \leq 0.01$ (n = 75 crosses per group).


F1
CTLM/CTLF
CTLM/GEMF
GEMM/CTLF
GEMM/GEMF
Duration of chase, nudge and lead (sec)
Duration of lateral and quiver (sec)
600
400
200
40
20
0
6
4
2
0
Chase
Nudge
Lead
Lateral
Quiver
F2
CTLM/CTLF
CTLM/GEMF
GEMM/CTLF
GEMM/GEMF
Duration of chase, nudge and lead (sec)
Duration of lateral and quiver (sec)
600
400
200
40
20
0
6
4
2
0
Chase
Nudge
Lead
Lateral
Quiver

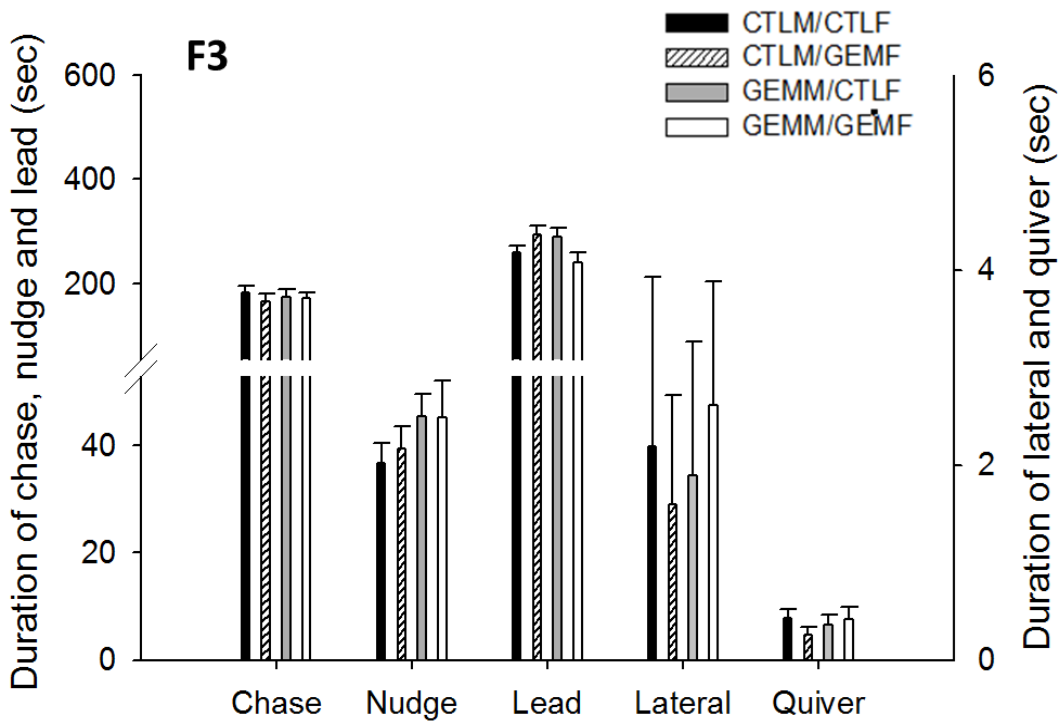
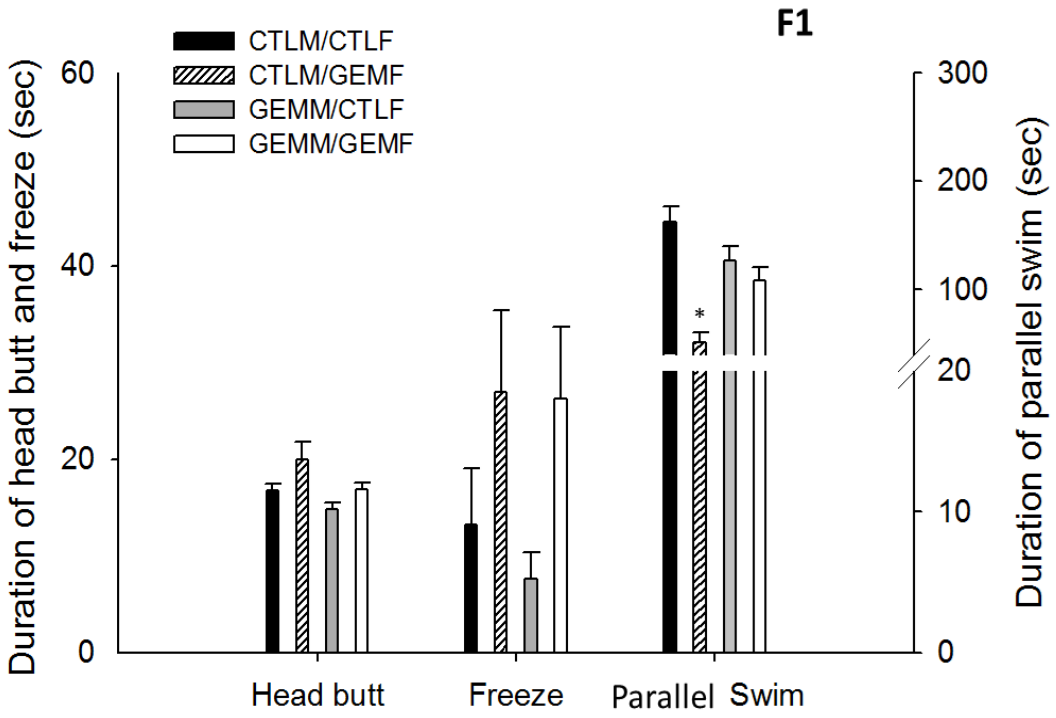
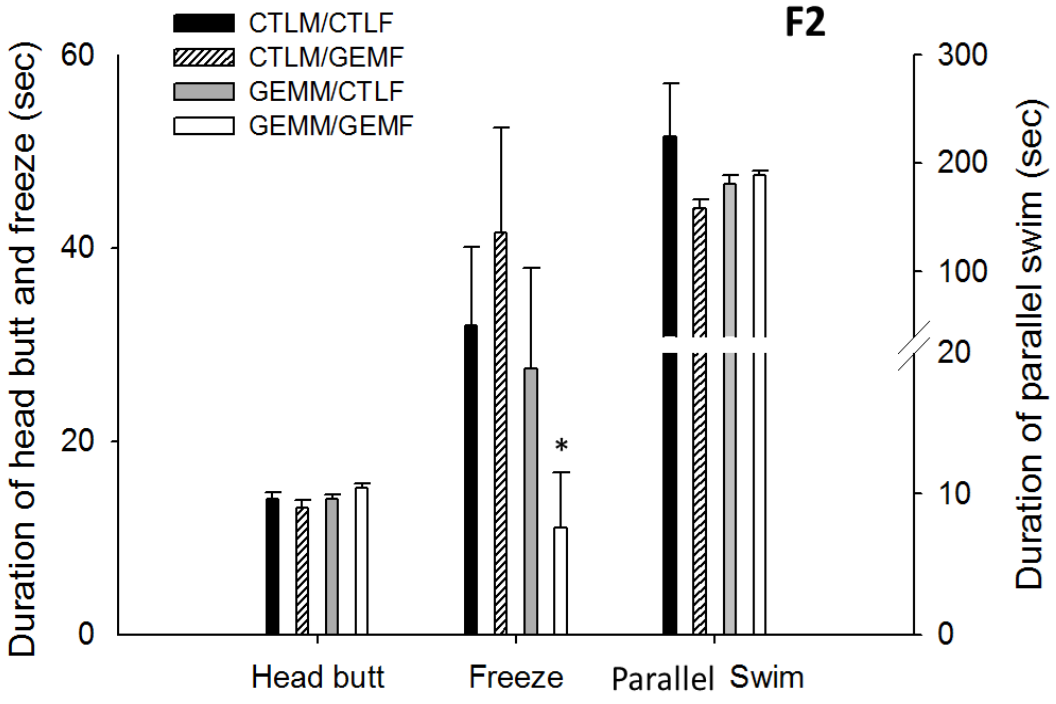


Figure 3.8: Time duration (mean ± SEM) of male courtship displays in unexposed zebrafish offspring from four lineages. Panels show data from F_1, F_2 and F_3 generations respectively. Figures legends indicate lineages generated by crossing unexposed (CTL) and gemfibrozil exposed (GEM) male (M) and female (F), F_0 zebrafish; F_1–F_3 generations were crossed within each lineage only (see section 3.2.3). Courtship videos were taken after first light, when pairs of male and female from the same lineage were crossed together. Videos were scored blind to determine frequency of five behaviours (see materials and methods for descriptions). Asterisks indicate significant differences from the respective controls, Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, (nF_1 = 38–42, nF_2 = 33–35, nF_3 = 40 videos per group).


F1
CTLM/CTLF
CTLM/GEMF
GEMM/CTLF
GEMM/GEMF
Duration of head butt and freeze (sec)
Duration of parallel swim (sec)
0
20
40
60
0
10
20
100
200
300
*
Head butt
Freeze
Parallel Swim


F2
CTLM/CTLF
CTLM/GEMF
GEMM/CTLF
GEMM/GEMF
Duration of head butt and freeze (sec)
Duration of parallel swim (sec)
0
20
40
60
0
10
20
100
200
300
*
Head butt
Freeze
Parallel Swim

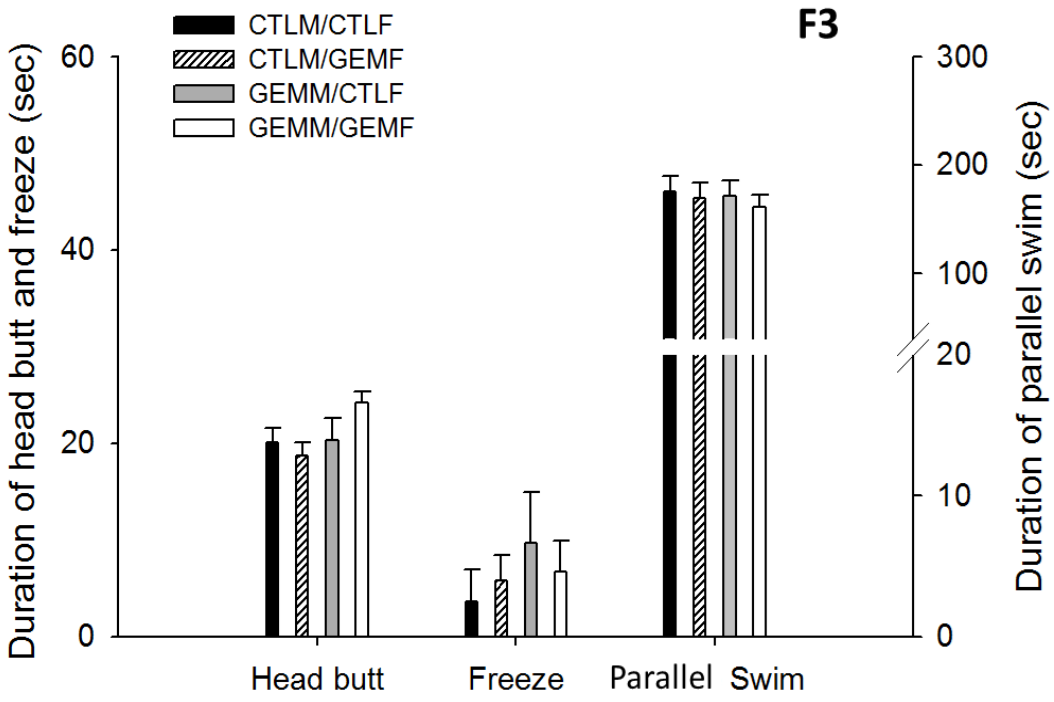


Figure 3.9: Time duration (mean ± SEM) of male aggressive displays (head butt, parallel swims) or anxiety (freeze) in unexposed zebrafish offspring from four lineages. Panels show data from F_1, F_2 and F_3 generations respectively. Figures legends indicate lineages generated from crosses of unexposed (CTL) and gemfibrozil (GEM) exposed male (M) and female (F) F_0 zebrafish; F_1–F_3 generations were crossed within each lineage only (see section 3.2.3). Videos of mirror interactions were taken from male fish belonging to the four lineages and were scored blind to determine duration of three behaviours (see materials and methods for descriptions). Asterisks indicate significant differences from the controls, Kruskal-Wallis ANOVA with Dunn's test; $p \leq 0.05$, (nF_1 = 37–41, nF_2 = 31–39, nF_3 = 30–41videos per group).

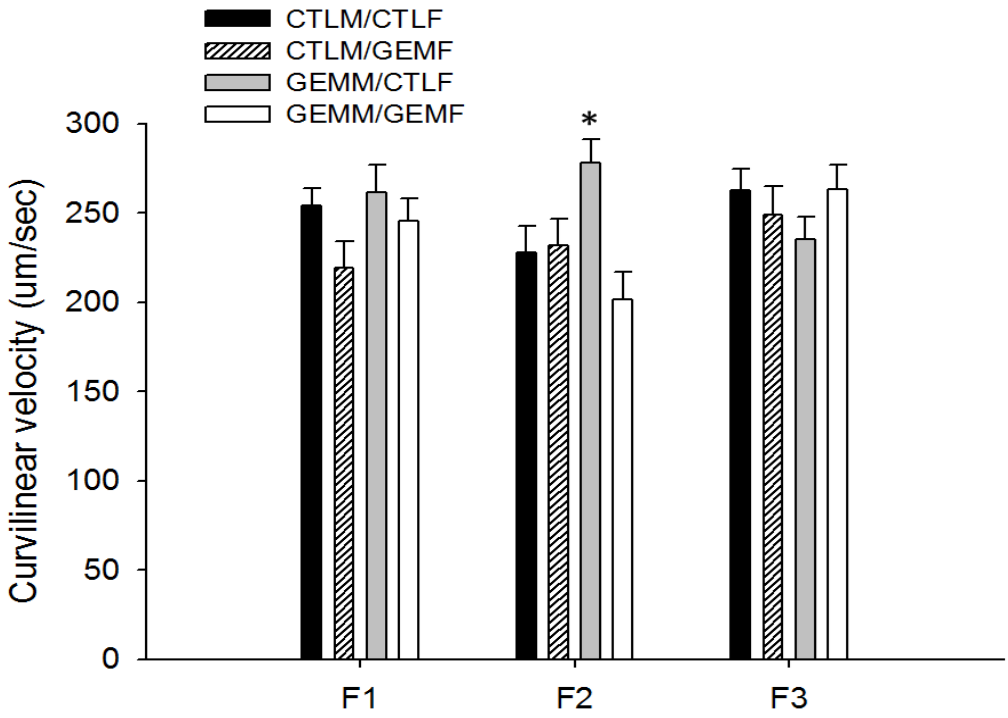


Figure 3.10: Curvilinear velocity (mean ± SEM) of zebrafish sperm at 20 sec post activation in unexposed male offspring from four lineages of F_1, F_2 and F_3 generations. The figure legend indicates lineages generated with F_0 males (M) and females (F) from unexposed (CTL) and gemfibrozil (GEM) exposure treatments; F_1–F_3 generations were crossed within each lineage only (see section 3.2.3). Milt samples were collected from male fish, activated with system water, and videotaped using Astro IIDC recording software and Olympus CX41 light microscope mounted with Prosilica EC-650 digital camera at 200x magnification, with subsequent analysis of edited video clips to assess sperm velocity through Ceros computer assisted sperm analysis. Asterisk indicates differences from controls, Linear model; $p \leq 0.05$, ($n = 22$ fish per group).

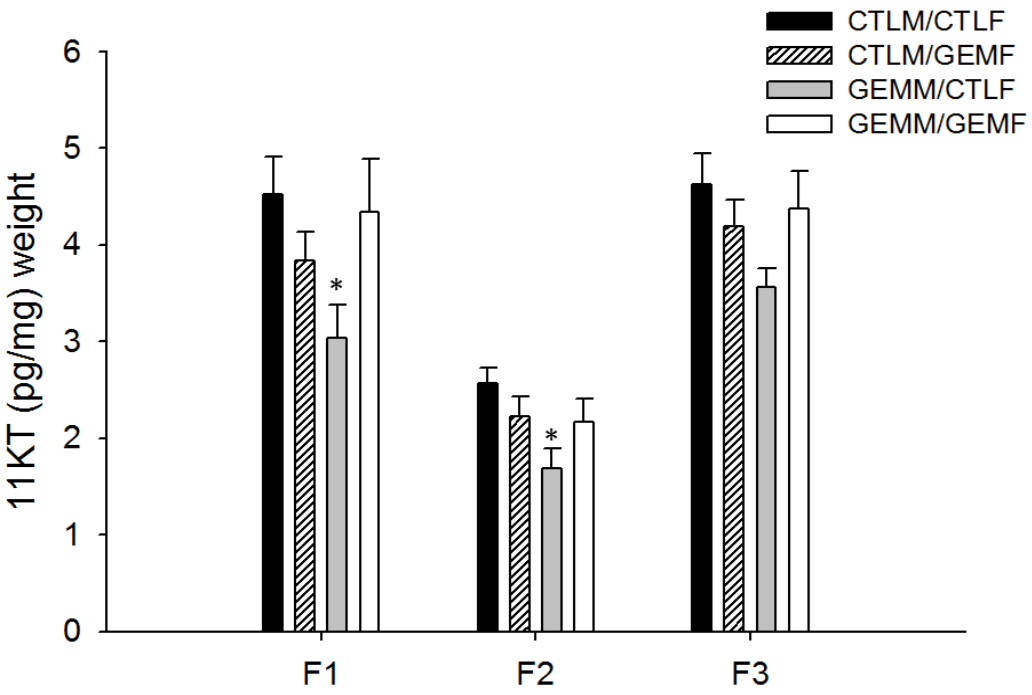
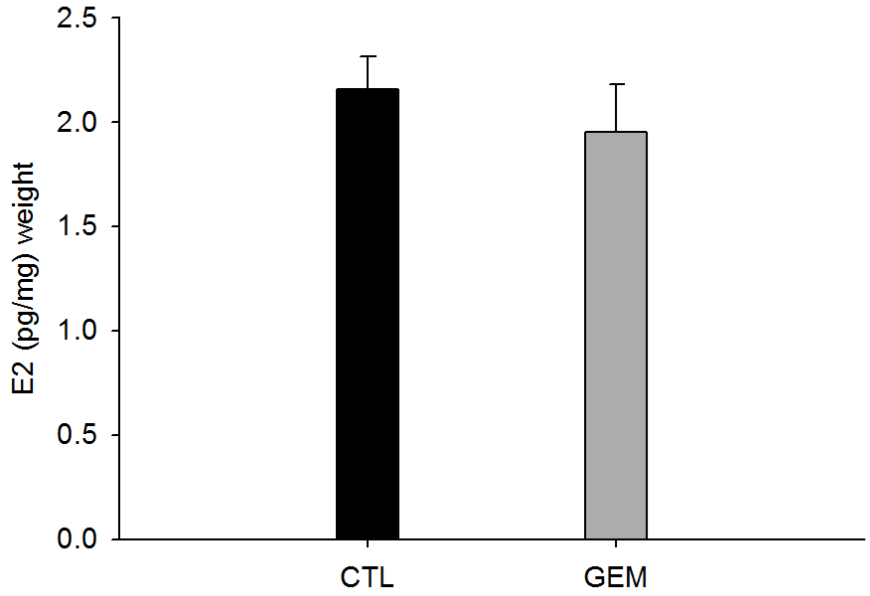


Figure 3.11: Levels of 11-Ketotestosterone (mean ± SEM) in whole body homogenates of unexposed zebrafish male offspring from four lineages of F_1, F_2 and F_3 generations. Figure legend indicates lineages generated by breeding unexposed (CTL) and exposed (GEM) male (M) and female (F) F_0 zebrafish; F_1–F_3 generations were crossed within each lineage only (see section 3.2.3). Asterisks indicate significant differences from the controls, ANOVA, with Holm-Sidak test, $p \leq 0.05$, (nF_1 = 15–18, nF_2 =15, nF_3 = 20).



Suppl. figure 3.1: Levels of estradiol (mean ± SEM) measured in whole body of F_0 zebrafish females exposed to 10 µg/L of gemfibrozil (GEM). CTL is unexposed animals. ANOVA with Holm-Sidak test, $p \leq 0.05$, (nCTL = 15, nGEM = 18).

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CHAPTER 4

GEMFIBROZIL AND CARBAMAZEPINE DECREASE STEROID PRODUCTION IN ZEBRAFISH TESTIS *(Danio rerio)*

Shamaila Fraz, Abigail H. Lee, and Joanna Y. Wilson

ABSTRACT

Gemfibrozil (GEM) and carbamazepine (CBZ) are two environmentally relevant pharmaceuticals and chronic exposure of fish to these compounds leads to decreased androgen levels and fish reproduction. The main focus of this study was to examine the effects of GEM and CBZ on testicular steroid production, using zebrafish as a model species. Chronic exposure to 10 µg/L GEM and CBZ decreased reproductive output of zebrafish and lowered whole body, plasma, and testicular 11-ketotestosterone (11KT). Testicular production of 11KT was examined post exposure using ex vivo cultures to determine basal and stimulated steroid production. The goal was to ascertain the step impaired in the steroidogenic pathway by each compound. Ex vivo 11KT production in testes from males chronically exposed to GEM and CBZ was lower than that from unexposed males. Although hCG, 25-OH cholesterol, and pregnenolone stimulation increased 11KT production in all treatment groups over basal levels, hCG stimulated 11KT production remained significantly less in testes from exposed males compared to controls. 25-OH cholesterol and pregnenolone stimulated 11KT production was similar

between GEM and control groups but the CBZ group had lower 11KT production than controls with both stimulants. We therefore propose that chronic GEM and CBZ exposure can reduce production of 11KT in testes through direct effects independent of mediation through HPG axis. The biochemical processes for steroid production appear unimpacted by GEM exposure; while CBZ exposure may influence steroidogenic enzyme expression or function.

4.1 INTRODUCTION

Pharmaceuticals constitute a class of biologically active, surface water contaminants of concern. Municipal waste water effluents serve as point sources for active pharmaceutical ingredients (APIs) and their metabolites to reach surface water (Ankley et al., 2007; Zhang et al., 2008). Fish living in receiving water may experience inadvertent effects, including endocrine disruption, due to long term/life time exposure to these compounds (Corcoran et al., 2010, Fent et al., 2006). Gemfibrozil (GEM) and carbamazepine (CBZ) are two APIs frequently detected in surface water across the globe (Santos et al., 2010; Gavrilescu et al., 2015; Kostich et al., 2014). These are generally detected at high ng/L – low µg/L in treated municipal waste water, and below 1 µg/L in surface water, because of their substantial use, incomplete removal thorough treatment processes and continuous release into surface water (Zhang et al., 2008; Gavrilescu et al., 2015; Kostich et al., 2014; Andreozzi et al., 2003; Fent et al., 2006; Koné, et al., 2013). These compounds have long degradation half lives in surface water and bioconcentrate in cultured and wild caught fish species (Araujo et al., 2011; Andreozzi et al., 2003;

Ramirez et al., 2009; Wang et al., 2011; Zenker et al., 2014). Chronic low dose exposure of adult zebrafish to CBZ and GEM decreased reproductive output and produced histological changes in the ovaries and kidneys; CBZ decreased plasma 11KT and GEM altered liver histology (Galus et al., 2013). GEM inhibited production of testosterone in pre exposed goldfish testes fragments in vitro (Cameron, 2011).

The potential for APIs to affect non target aquatic species through conserved molecular targets has been the focus of recent ecotoxicological assessments (Hugget et al., 2003; Gunnarsson et al., 2008; Lissemore et al., 2006; Gavrilescu et al., 2015; Ankley et al., 2007). GEM is as a peroxisome proliferator activated receptor alpha (PPARα) ligand in mammals from the fibrates class of lipid regulators. It is used to lower plasma triglycerides, total cholesterol and high density lipoproteins by regulating biosynthesis and breakdown of cholesterol and fatty acid oxidation in humans (Staels et al., 1998). Long term treatment is linked to reversibly reduced libido and impotence in male patients (Rizvi et al., 2002). In rat Leydig cells, GEM exposure reduced basal and human chorionic gonadotropin (hCG) stimulated production of testosterone in vitro (Liu et al., 1996). CBZ acts by inhibiting voltage gated sodium and calcium channels, but its exact mechanism of action is complex and unclear. It is primarily used for epilepsy treatment in humans (Ambrósio et al., 2002). Long term treatment of men is associated with adverse effects like lower circulating testosterone levels due to decreased testicular biosynthesis and increased synthesis of sex hormone binding globulin (SHBG, Isojärvi et al., 1995; Isojärvi et al., 2004; Taubøll et al., 2008). In vivo exposure of male rats with CBZ

reduced plasma testosterone and decreased testosterone production in isolated rat Leydig cells cultured in vitro (de Oliva & Miraglia, 2009; Kühn Velten et al., 1990).

Though only a few studies investigated the effects of GEM on fish reproduction to date, the negative effects of fibrates on fish reproduction are well known (Al-habsi et al., 2016; Runnalls et al., 2007; Velasco-Santamaría et al., 2011; Coimbra et al., 2015). Fourteen day waterborne exposure of male goldfish to 1.5 and 1500 µg/L GEM reduced circulating testosterone, and 96 hour acute exposure to 1500 µg/L reduced expression of steroidogenic acute regulatory (StAR) mRNA in testes (Mimeault et al., 2005). Moreover, pre-exposure of goldfish testicular fragments with 100µM GEM inhibited testosterone production in vitro (Cameron, 2011). Previously, our lab showed that chronic six weeks separate waterborne exposures of adult zebrafish to 0.5 and 10 µg/L GEM and CBZ reduced mean embryo production, with significantly lower plasma 11-ketotestosterone (11KT) in males exposed to 0.5 µg/L CBZ (Galus et al., 2013; 2014). These exposures at either test concentrations caused no changes in plasma estradiol in females (Galus et al., 2013). To determine the potential negative effects of GEM and CBZ on 11KT in zebrafish, the current study investigated the hypothesis that chronic exposure to 10 µg/L GEM and/or CBZ would adversely affect the male endocrine system and reduce 11KT by means of reduced testicular production. We assessed this hypothesis by examining 11KT in plasma, whole body, and testes after chronic exposure and by employing ex vivo testis culture to assess basal and stimulated 11KT production in tissues from pre-exposed zebrafish.

4.2 MATERIALS AND METHODS

4.2.1 Animal Housing

Adult wildtype zebrafish (*Danio rerio*) were purchased from AQUAlity Tropical Fish Wholesale Inc. (Mississauga, Canada). Male and female fish were sorted through visual identification of secondary sex characteristics and relocated in a multi-rack holding system (AHAB, Pentair Aquatic Ecosystems Inc. USA) at a density of three fish per liter and equal male to female ratio. The water chemistry was maintained by automatic dosing of distilled water with sodium bicarbonate (Aquatic Eco systems Inc. USA) and Instant Ocean sea salts (Instant Ocean salts, Spectrum brand, USA). Water renewal was 10% daily. The pH was 6.8 – 8.0 and the conductivity was 300 – 400 µS/cm. The water was maintained between 27 – 29°C and aerated to >80% dissolved oxygen. Fish were kept on a 14:10 h day night cycle and were fed tropical fish flakes (Nutrafin Max tropical fish flakes, Hagen, USA) twice a day, and live adult brine shrimps (*Artemia nauplii;* Atremia Cysts, Inve Aquaculture Nutrition, USA) once a day.

Every day for 15 days before exposure, breeding output was monitored in each tank by placing an embryo trap (rectangular food grade polypropylene containres fitted with netted lids) into the bottom of the tank each morning before first light. The traps were withdrawn from the tanks 1.5 hours after first light and embryos were counted. At the end of the 15 day breeding output assessment period, tanks with comparable reproductive output were randomly selected to make three replicate tanks for each of the three treatment groups i.e. control (CTL), gemfibrozil (GEM) and carbamazepine (CBZ).

Fish care and experimental procedures were approved by the McMaster Animal Ethics Review Board under the Animal Utilization Protocol # 16-09-34 to JYW.

4.2.2 Exposure Conditions

Fish were transferred from the multi-rack system to stand-alone glass aquaria and habituated for 3 days before exposure; fish were at a density of 3 fish/L and 50:50 sex ratios. Each of the three treatments was tested in triplicate glass aquaria i.e. solvent control, 10 µg/L of gemfibrozil, and 10 µg/L of carbamazepine. All tanks were kept at the same water quality parameters as the housing system and had filter pumps to recirculate water. Temperature and pH were monitored daily and weekly monitoring of conductivity, dissolved oxygen (using portable YSI, YSI Inc. USA), nitrate, nitrite, general hardness, carbonate hardness and ammonia were conducted (Nutrafin Test, Hagen, USA). Dosing solutions of carbamazepine and gemfibrozil (Sigma Aldrich, Canada) were prepared in DMSO (Caledon Lab Chemicals, USA) and added to the exposure tanks to obtain nominal concentration of 10 µg/L; the control tanks were dosed with DMSO. The final concentration of DMSO in all the tanks (CTL, GEM and CBZ) was 0.004%. During the exposure period, water quality was maintained by 90% change out every third day with dose renewal. The method of stocks preparation and storage, dosing interval, and water renewal were analogous to methods in Galus et al., 2013. A schematic of the exposure and sampling is provided in fig. 4.1 (top panel).

4.2.3 Exposure for Reproductive Assessments

4.2.3.1 Whole Tank Breeding

Reproduction in CTL, GEM and CBZ triplicate tanks was monitored daily by whole tank matings from day 1–42 of the exposure (Fig. 4.1, top panel), as described above, and in Galus et al., 2013. The re-circulating filters were turned off during embryo collections. Embryos were removed after 1.5 hours, drained, and rinsed with E3 embryo medium (NaCl = 292 mg/L, KCl = 2.83 mg/L, $MgSO_4$ = 5.5 mg/L, $CaCl_2$ = 5.5 mg/L), counted under a microscope, and then transferred to 120 x 20 mm glass petri dishes at a density of 150 embryos per dish in an incubator at 28°C for six hours. At six hours post fertilization, the total number of embryos was counted, including viable, unfertilized eggs and non-viable (dead at 6 hours post fertilization). We report fecundity in whole tank breeding as the mean viable embryos produced per female per day. After 42 days, non-invasive testing of the exposed animals was conducted (data not shown here) until day 67 when the experiment was terminated and plasma, gonad, and whole bodies were sampled for steroid analyses. Only the reproductive assays (day 1–42) and steroid analyses (after 67 days exposure) are included here.

4.2.3.2 Sex Steroids

Male fish were sampled by identification of secondary sex characters, euthanized, blotted dry, weighed, and frozen in liquid nitrogen for whole body steroid measurements. Extractions were performed as outlined by Alsop & Vijayan, 2008. Whole frozen fish were homogenized using a tissue homogenizer (GLH tissue homogenizer, Omni

International) for 60 sec with 5 times w/v of ice cold nanopure water. 0.5 ml of each fish homogenate was extracted three times repeatedly, with 8 volumes of diethyl ether (Fisher, Canada). The ether extracts from one fish were combined and evaporated until dry in a water bath at 37^{o}C, followed by air drying for 2 hours and resuspension in 0.5 ml ELISA buffer (Cayman Chemical, Ann Arbor, USA). The suspensions were kept at 4^{o}C with occasional vortexing for 12 hours and stored at -80^{o}C for future use. Testes were extracted in identical manner and the extracts were re-suspended in 0.1 ml of ELISA buffer. The extracts were used to determine the amount of total 11KT, as processing of samples thorugh the organic solvent extractions would release free 11KT. The extraction efficiency was between 76 – 80%. To separate plasma, blood from a single fish was collected in a heparinized capillary tube (Fisher, Canada) through caudal incision, transferred to a heparinized microfuge tube, and centrifuged at 12,000 x G for 10 minutes at 4^{o}C. The plasma supernatant was transferred to a new tube and stored at -80^{o}C for further use. The plasma samples were used directly in the assay to get the amount of free 11KT (unbound to plasma proteins). 11KT was analyzed in duplicate wells using a colorimetric 96-well ELISA kit (Cayman Chemical, Ann Arbor, USA) for plasma, whole body or testes. The experimentally determined limit of detection for 11KT assay was 1.37 ± 0.23 pg/ml (mean ± S.D), and inter and intra-assay coefficients of variation were less than 18% and 5% respectively. The experimentally determined cross reactivity of 11KT ELISA kit for testosterone was 0.016 %.

4.2.4 Pre-exposure for ex vivo Testes Culture

Fish were purchased, housed and maintained by identical methods described in section 4.2.1, and pre-exposure breeding assessment was carried out as previously described. The exposure conditions, stock concentrations, dose renewal regimen were maintained as described in section 4.2.2.

4.2.4.1 Whole Tank Breeding

We had previously determined in four repeated experiments that a 6 weeks water borne exposure to 10 µg/L GEM and CBZ reduced fecundity and this effect plateaus after 4 weeks of exposure (Galus et al., 2013; 2014). Therefore, reproductive output (cumulative mean embryo production per tank as described in section 4.2.3.1) was determined only in weeks 5 and 6, to confirm that exposures were producing similar biological effects on reproduction. At the end of exposure, males were dissected to collect testis, which were cultured ex vivo (described in section 4.2.4.2). A schematic of the exposure and sampling is provided in Fig. 4.1 (lower panel).

4.2.4.2 Testes Tissue Culture

Testes tissue culture technique was adapted from Leal et al., 2009 with few modifications. Basal medium was prepared by supplementing Leibovitz L-15 medium (Life technologies, Canada) with 10 mM HEPES (Life technologies, Canada), 0.5% w/v bovine serum albumin fraction V (Sigma Aldrich, Canada), 10 nM retinoic acid (Sigma Aldrich, Canada), 0.4 mg/L amphotericin B (Life technologies, Canada), 200 U/ml penicillin and 200 µg/ml streptomycin (Penicillin-streptomycin solution from Sigma

Aldrich, Canada). The pH of medium was adjusted to 7.4, filter sterilized and stored at 4^{o}C.

The original method involved mounting the testis on top of an assembly composed of an agar cylinder and a piece of nitrocellulose membrane (150µm thickness, 0.22 µm pore size, 0.25 cm2) placed in 1 ml of the incubation medium, in each well of the 24 well plate. The technique was optimized based on four experiments. A) The difference between the release of 11KT directly in culture media versus the original assembly on agar/nitrocellulose was assessed; 11KT levels were higher without the agar/nitrocellulose. Media was spiked with 11KT with and without agar/nitrocellulose and levels of 11KT were found to be less with the agar/nitrocellulose suggesting that the hormone was taken up/retained by the agar, as shown by Leal et al. (2009). Thus, we decided to directly culture testes in media without the agar/nitrocellulose. B) The organic solvent extraction from the media was determined; our experimental extraction efficiency was 41 – 43 % which was in agreement with Leal et al. (2009) who reported an extraction efficiency of close to 40 %. C) The direct use of medium for EIA without extraction was assessed and found to give a higher (21%) recovery of 11KT relative to the medium with extraction. This was expected since our organic solvent extraction efficiency was between 76–80%. D) The appropriate volume of medium (0.5 versus 1 ml/well) was assessed and 0.5 ml was found to complement with the short incubation time of 3 hours in our experiments. Thus, we incubated the organ in 0.5 ml media per well without the agar/nitrocellulose insert and used the medium directly for EIA analysis.

Three types of stimulated media were tested i.e. human chorionic gonadotropin (hCG), 25-hydroxy cholesterol and pregnenolone. All the stimulators were purchased from Sigma Aldrich, Canada. In order to aid the penetration of water soluble steroidogenic stimulants like hCG into the tissue, the effect of gentle pricking (3 –5 pricks per testis) was determined. The stimulation of testis with 10 IU/ml hCG produced 11–19 fold or 20-30 fold increase in 11KT release over basal controls without or with gentle pricking, respectively. Nonetheless pricking had limited effect on the penetration of hydrophobic stimulants. 50μM cholesterol stimulated the steroidogenic response to 6 – 13 and 10 –18 folds above the basal controls for testes with and without pricking, respectively. Incubations with 100nM pregenenolone with and without pricking stimulated the steroidogenic response to 9 – 19 and 12 – 21 fold, respectively above the basal controls. Concentrations of stimulants were assessed at two concentrations (10 and 100 IU/ml hCG, 50 and 100 μM 25-hydroxy cholesterol, and 100 and 200 nM pregnenalone) to determine if increased levels, compared to previously published concentrations, induced significantly higher 11KT release; based on this, final concentrations of 10 IU/ml hCG, 50μM 25-hydroxy cholesterol and 200 nM pregnenolone were chosen.

hCG is a ligand of fish LH receptors, 25-hydroxy cholesterol is a permeable form of cholesterol that does not need StAR mediated transport across the mitochondrial membrane, and pregnenolone is formed from cholesterol by the action of P450-side chain cleavage enzyme (P450-SCC also called CYP11a) and is the first and the common precursor to all steroid hormones. The stimulants were chosen to examine whether GEM

or CBZ exposure was interfering in the early stages of the steroidogenic pathway or whether the later biochemical process was influenced. This was based on the assumption that exposed testes would have lower steroid production than controls and if steroidogenic inhibition in pre-exposed explants was reinstated by a certain stimulator, the prior step would be considered impacted by the pre-exposure (Fig. 4.2).

Under sterile conditions, male fish from the exposure tanks (CTL, GEM and CBZ) were euthanized, testes were removed and transferred into clean petri dishes containing basal Leibovitz L-15 medium. Shortly after, the two testes were separated and transferred to individual wells of a 24 well plate, where each well contained 0.5 ml of basal or stimulated medium. The tissue was gently pricked with sterile needles carefully enough not to fragment the testes. From each fish, one testis (right/left) was randomly selected to serve as a basal control for the contralateral testis from the same fish, which was treated with the medium spiked with stimulators of steroid production (Leal et al., 2009). The average weight of testis explants ranged from 2.5 –11 mg. The explants were incubated in humidified air at 28 ± 0.2^{o}C for three hours, followed by medium collection and storage at -80^{o}C until the measurement of 11KT by ELISA. Five male fish were sampled from each of the triplicate tanks belonging to each treatment group (CTL, GEM and CBZ), for a total sample size of 15 fish per experimental condition (hCG, 25-OH cholesterol and pregnenolone).

4.2.5 Chemical Analysis

Samples for chemical analysis (10 ml) were collected at 10 min after water change out during the 6 weeks exposure period, covered in foil and stored at -20 ^{0}C. The water change was randomly selected from all possible water changes in the experiment. All the extractions and analysis were performed at Water Quality Center, Trent University. Samples were prepared by a "dilute and shoot" technique. 100 µl of sample was diluted with 100 µl of methanol and spiked with 10 µl of a 1 ppm solution of both D10-carbamazepine and D6-gemfibrozil in methanol. Analysis was conducted by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) using an AB Sciex Qtrap 5500 mass spectrometer coupled with a Shimadzu 10A liquid chromatograph instrument, and Perkin Elmer Series 200 autosampler. A Thermo Acclaim RSLC120 C18 column (2.2 µm, 4.6 x 150 mm) was employed to separate the analytes. Methanol and 20 µM ammonium acetate in milli-Q water were used as mobile phases operated with gradient elution and a flow rate of 0.25 mL/min. Injection volume was 10 µl. Gemfibrozil was analyzed in negative ion mode while carbamazepine was analyzed in positive ion mode. Samples were analyzed in multiple reactions monitoring mode (MRM), with two precursor-product ion transitions for each analyte. Samples were quantified with internal calibration of the isotopically labelled standard, using a seven point calibration curve. The method limit of quantitation (LOQ) was 0.5 µg/L.

4.2.6 Statistics

Cumulative mean embryo production was normalized to the number of females per tank, which was determined by dissections at the end of the experiment. Means were

based on triplicate tanks. The data was analyzed by Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$ to compare the groups with the control. The amounts of 11KT were calculated by normalizing either to total body weight for whole body measurements, or to the weight of a pair of testes from individual fish to determine gonadal levels. The data for 11KT measurements in plasma, whole body and testicular samples were analyzed by ANOVA and Holm-Sidak post hoc test ($p \leq 0.05$), to detect differences from the controls. The amount of 11KT released in the culture medium from the ex vivo tissue culture experiments was determined by normalizing to the weight of individual testis. Paired observations i.e. basal versus stimulated from each of three treatment groups CTL, GEM and CBZ were analyzed by paired t-test with Bonferroni correction $p \leq 0.025$. One way ANOVA was used for comparison of three groups (basal/stimulated from CTL, GEM and CBZ); with Holm-Sidak post hoc test, $p \leq 0.05$. All analyses were conducted using Sigma Plot 10.0 (Systat Software Inc).

4.3 RESULTS

The actual aqueous tank concentrations of GEM (mean ± STD) and CBZ were 17.5±1.78 and 11.2±1.08 µg/L respectively. Reproductive output was significantly decreased in response to GEM and CBZ exposures in each of the two exposure experiments. GEM exposure led to a 44% and 60% decline in fecundity in experiment 1 and 2, respectively (Fig. 4.3). CBZ exposure caused a 48 % and 68% reduction in fecundity as compared to the controls, in experiment 1 and 2, respectively (Fig. 4.3).The levels of 11KT in males

exposed to GEM and CBZ were significantly less than the controls regardless of whether it was whole body, plasma or testes samples (Fig. 4.4).

4.3.1 11-Ketotestosterone production ex vivo

After six weeks pre exposure of fish, testes explants were incubated in medium, ex vivo, and the amount of 11KT released in the medium was measured. Basal release of 11KT was on average from 0.0125±0.0005 ng/mg tissue for testes from unexposed males; the explants collected from fish pre exposed to both GEM and CBZ released significantly lower levels of 11KT in the medium, as compared to the control fish (Fig. 4.5). Stimulation of testes by hCG significantly increased 11KT production in all treatment groups, over basal levels. Likewise, 25-OH cholesterol (Fig. 4.6) and pregnenolone (Fig. 4.7) significantly increased 11 KT productions, over basal levels in all treatment groups.

Production of 11KT, after stimulation with hCG, was significantly less for testes from GEM exposed males, compared to testes from control males (Fig. 4.5). While the production of 11KT, after stimulation with 25-OH cholesterol and pregnenolone, was comparable for testes from GEM and control males (Fig. 4.6, 4.7). The production of 11KT remained lower in testes from CBZ exposed males, compared to controls, regardless of whether they were stimulated by hCG (Fig. 4.5), 25-OH cholesterol (Fig. 4.6) or pregnenolone (Fig. 4.7).

4.4 DISCUSSION

Chronic exposure of zebrafish to 10 µg/L GEM and CBZ reduced fecundity (Fig. 4.3) and reduced 11KT in whole body, plasma and in testes of male fish (Fig. 4.4).

Changes in hormone levels provide useful information since alterations could be linked with perturbations of the physiological process regulated therein (Archand-Hoy & Bensen, 1998). Thus, we decided to examine 11KT production ex vivo after identifying negative impacts on a range of male specific endpoints (sperm velocity, sperm morphology, male courtship behaviour and male aggression – Chapter 2, Chapter 3); and confirmed an impairment of testicular steroidogenesis ex vivo in males chronically exposed to GEM and CBZ. The production of 11KT from the testes of exposed animals was examined after stimulation with step specific steroidogenic stimulators such that restoration of the steroidogenic response with a stimulant was taken as impairment of the step prior to the one activated by the stimulator. Human chorionic gonadotropin (hCG) is a ligand of fish LH receptors which was probed to test the possibility of lower sex hormone production due to reduced gonadotropin stimulation in vivo; if basal but not hGG stimulated 11KT production was lower in treated testes, we could assume that the hypothalamic-pituitary-gonadal (HPG) signaling was disrupted in the male fish in vivo. 25-OH cholesterol is a readily permeable form of cholesterol which was used to test the hypothesis of impaired cholesterol transport in the mitochondria. If basal but not 25-OH stimulated 11KT production was lower in treated testes, we could assume that HPG axis signaling was intact, but that the relay of that signal to move cholesterol into the mitochondria for steroidogenesis was impaired. In this case, second messenger systems or StAR regulation or function would be implicated. Pregnenolone is the common precursor to all steroid hormones, formed from cholesterol by the action of P450-side chain cleavage (P450-SCC); therefore, probing this step could indicate altered expression

or activity of P450-SCC. Lastly, if 11KT production was lower in treated testes with all stimulators, it would suggest that there was an impact on the expression or activity of steroidogenic enzymes after P450-SCC.

Fish reproduction is an ecologically relevant parameter included in regulatory testing guidelines (reviewed by Manibusan & Touart, 2017). We have included fecundity assessments since it is a population level endpoint and an accredited functional measure of the reproductive performance (OECD, 2008). Fecundity is widely considered a measure more related to female reproductive output, (e.g. reduced production or quality eggs) but lower fecundity may be related to altered male reproductive traits such as decreased courtship (Galus et al., 2013; Bobe & Labbé, 2010). A significant decline in embryo production after six weeks waterborne exposure of zebrafish to 10µg/L each of GEM and CBZ was found in both experimental exposures. Our findings corroborate with former studies, which reported significant reductions in fecundity in response to identical exposures to 10 µg/L GEM and CBZ, and to concentrations of 0.5 µg/L as well (Galus et al., 2013; 2014). Similarly, a modest decline in fecundity after GEM exposure was found in fathead minnows (Skolness et al., 2012). Negative reproductive effects of other fibrates, including clofibric acid in fathead minnows and bezafibrate in zebrafish have been documented (Runnals et al., 2007; Velasco-Santamaría et al., 2011). Further, gender derived adverse effects on male rat fertility due to CBZ treatment have been frequently reported in the mammalian literature (Fitzgerald et al., 1987; de Oliva & Miraglia, 2009; Andretta et al., 2014; Osuntokun et al., 2017).

We measured 11KT in whole fish, plasma and testes collected after 67 days of exposure (Fig. 4.3). Both testosterone and 11KT are present in high concentrations in teleost fish plasma (Borg et al.,. 1994), but the dominant androgen depends on physiological factors like species, male reproductive tactics, changes due to seasonal/spawning cycle and developmental stage (Borg et al., 1994). 11KT shows distinct sex specific changes in many teleost fish, since circulating levels are significantly higher in males than the females e.g. zebrafish, winter flounder, three spined stickleback, rainbow trout, and African catfish (as reviewed in Borg et al., 1994; Tokarz et al., 2013). It is the most potent, active androgen in zebrafish males and a ligand to androgen receptors (Toakrz et al., 2015).

Whole body 11KT measurements were chosen to complement the findings of individual fish plasma analysis for two major reasons. First, zebrafish are small animals from which only a very small plasma volume can be collected. Thus, sufficient plasma can not be collected from all individuals in an experiment and limited sample size can impact the statistical power in data analyses. Whole body steroid analyses would always be possible in an individual animal, and the organic solvent extraction could free the protein bound hormone so as to provide measurement of total hormone. Second, plasma concentration provided measurement of only free circulating hormone, as plasma samples were assayed directly without organic solvent extraction. Plasma concentration could not give a quantity of total bioavailable fraction or total hormone; and is therefore not a good indicator of androgen deficiency (Matsumoto & Bremner, 2004; Rosner et al., 2007; Ly & Handelsman, 2005). Testicular analyses was employed to determine if reduced 11KT

production was likely the cause of lower circulating 11KT levels, as opposed to alteration in bioavailable androgen and/or rate of hepatic metabolism.

We found significantly lower 11KT levels in plasma, whole body, and testes of males exposed to GEM and CBZ as compared to the controls (Fig. 4.4). Similar observations have been reported by other studies. Chronic exposures to GEM reduced serum testosterone in male goldfish (Mimeault et al., 2005). Exposure of zebrafish to another fibrate lipid regulator, bezafibrate, reduced circulating 11KT in males (Velasco-Santamaría et al., 2011). Moreover, a modest decline due to GEM, while significant drop due to CBZ, occurred in plasma 11KT of zebrafish males after six weeks exposures (Galus et al., 2013). CBZ produced similar effects in rodents; male rats showed reduced circulating testosterone levels when exposed to CBZ in vivo (de Oliva & Miraglia, 2009; Al-Sanafi et al., 2013; Andretta et al., 2014). Collectively, these data suggest that both CBZ and GEM impact testicular androgen production after chronic exposure to zebrafish. We directly tested this hypothesis using ex vivo testes cultures to assess 11KT production in testes from male zebrafish chronically exposed to each pharmaceutical.

Steroid biosynthesis begins (Fig. 4.2) by binding of luteotropic hormone (LH), to G-protein coupled receptors in the cell membrane of steroidogenic cells. LH binding stimulates synthesis of lipids and proteins, and phosphorylation of proteins including StAR through cAMP protein kinase (Stocco & Clark, 1996). StAR acts as a transport protein to facilitate the entry of hydrophobic cholesterol into the mitochondria; this movement is the rate limiting step in steroid synthesis. Cholesterol is converted by P450-SCC to pregnenolone, which is transported out of mitochondria and serves as the

common precursor to biosynthesis of all steroids in the endoplasmic reticulum (Stocco & Clark, 1996). The later steps are regulated by the amount and/or activity of various enzymes involved in steroidogenesis e.g. CYP 17a, aromatase, 11β-HSD (Stocco & Clark, 1996).

A steroidogenic diminution was observed in GEM pre-exposed explants in basal/non-stimulated conditions (Fig. 4.5), which is indicative that steroid production in testes was impaired and at least partially accounted for the lower circulating 11KT in vivo. Reduced steroidogenic responses to hCG stimulation in pre-exposed explants implied that GEM might be interfering with cell signaling step later to the activation of receptors by LH (Cameron, 2011). The prospects include interaction with cAMP production/release, changes in StAR transcription or phosphorylation, and/or other signaling cascades (Cameron, 2011; reviewed by Stocco et al., 2005). Comparable to our study, GEM and fenofibrate reduced in vitro steroidogenic response to hCG stimulation in goldfish testis fragments pre exposed to these fibrates for 18 hours (Cameron, 2011). Bezafibrate exposure reduced steroidogenic response to hCG stimulation both in MA-10 mouse Leydig tumor cells and rat Leydig cells, cultured in vitro (Cameron, 2011; Liu et al., 1996; Gazouli et al., 2002).

11KT production was stimulated by incubation with 25-OH cholesterol, and sustained in the presence of pregnenolone (Fig. 4.6, 4.7), with no statistical differences between testes from control and exposed males. This strongly suggests our finding that fibrate exposure might impact cholesterol movement similar to that observed in goldfish and in MA-10 mouse Leydig tumor cells (Cameron, 2011; Gazouli et al., 2002). The

authors of these studies proposed fibrates to interfere with the cellular mechanisms between the release of cAMP and the cross mitochondrial transport of cholesterol (Cameron, 2011; Gazouli et al., 2002), as forskolin/dibutyryl cAMP were not able to stimulate steroidogenic response. Reduction in StAR mRNA and consequent substrate availability was suggested one of the possible mechanisms for the decline in plasma testosterone in male goldfish exposed to GEM (Mimeault et al., 2005). Low plasma cholesterol due to exposure of zebrafish males with bezafibrate was significantly correlated with StAR mRNA (Velasco-Santamaría et al., 2011). We therefore, support the hypothesis of probable impairment of cholesterol availability for the synthesis of steroids. Nonetheless, in zebrafish, the potential reduction in the levels of cAMP, which is the main secondary messenger signaling for transcription and phosphorylation of StAR, levels of total StAR protein/phosphorylated StAR, in addition to other signaling cascades need further investigation.

Hypolipidemic effects of fibrates were reported in zebrafish, grass carp and rainbow trout (Al-Habsi et al., 2016; Velasco-Santamaría et al., 2011; Du et al., 2008, Prindiville et al., 2011). It is unclear whether these effects in fish are manifested selectively by PPARα activation (Mimeault et al., 2006; Velasco-Santamaría et al., 2011). Fibrates including GEM bind preferentially to PPARα, with weak binding to PPARβ and PPARγ, in mammals and can exert actions independent of PPAR activation (Desvergne & Wahli, 1999; Roy & Pahan, 2009). All three types of PPARs (PPARα, PPARβ, and PPARγ) exist in various fish, but the roles in reproduction have not been studied (Ruyter et al, 1997; Leaver et al., 1998; Mimeault et al., 2006; Ibabe et al., 2002). Nevertheless,

fibrates (mammalian PPARα ligands) are implied to affect fish reproduction indirectly by interfering with biosynthesis and breakdown of cholesterol and fatty acids, and altering cholesterol availability for synthesis of sex steroids by reducing plasma cholesterol, which is the primary substrate for the synthesis of all steroid hormones (Velasco-Santamaría et al., 2011; Cameron, 2011; Manibusan & Touart, 2017). In zebrafish exposed to bezafibrate, lower plasma cholesterol was positively correlated with decreased plasma 11KT (Velasco-Santamaría et al., 2011). Similarly, exposure of zebrafish caused a pronounced reduction in whole body cholesterol in males (Al-habsi et al., 2016). We therefore suggest that altered plasma lipid profiles should be investigated as a possible mechanism by which fibrates may influence cholesterol availability in the testes.

The effect of pre-exposure to CBZ was evident in ex vivo testes culture, because the amount of 11KT released in the medium under basal/non stimulated conditions were considerably lower than control explants (Fig. 4.5, 4.6, 4.7). Stimulation of explants from CBZ pre-exposed fish with either of hCG, 25-OH cholesterol and pregnenolone did elicit increased 11KT production, compared to basal levels, yet remained lower than that seen in testes from control males (Fig. 4.5, 4.6, 4.7). The diminished steroidogenic response to hCG stimulation in CBZ treated testes explants is similar to the effects reported in rats Leydig cells in vitro (Kühn Velten et al., 1990), where CBZ decreased testosterone production in the response to stimulation with hCG. Contrary to this study, the inhibition of steroidogenesis was reinstated by incubation with 25-OH cholesterol and the authors proposed that CBZ could be acting at a site between release of cAMP and the transport of cholesterol to the inner mitochondrial membrane, since there were no differences in

steroids produced in response to hCG and dibutaryl cAMP stimulation (Kühn Velten et al., 1990). Nonetheless, we observed no restoration of the steroidogenic response in the presence of 25-OH cholesterol and pregnenolone. Thus, it may be implied that CBZ is inhibiting a step distal to the formation of pregnenolone in the steroidogenic pathway in zebrafish testes. This is supported by the reports that CBZ had no effect on the activity of CYP11a (commonly known as P450 SCC) in bovine adrenocortical mitochondria (Ohnishi & Ichikawa, 1997) and the expression of CYP11a and StAR mRNA in human adrenal carcinoma cell line H295R (Gustavsen et al., 2009). However, CBZ down regulated CYP17a and CYP11b mRNA in human adrenal carcinoma cell line H295R, and inhibited the activity of CYP11b more potently than CYP17a (Gustavsen et al., 2009). CYP17a is considered a bottle neck for the synthesis of 11KT while CYP11b regulates the formation of 11KT precursors at the final stages of steroidogenesis (Tokarz et al., 2015; 2013). Unfortunately, not much is known about specific effects of CBZ on the expression or activity of zebrafish steroidogenic genes and enzymes. This possibility could be further supported because antiepileptics are suggested to accumulate in cellular membranes like endoplasmic reticulum due to their hydrophobicity (Ohnishi & Ichikawa, 1997). In summary, we propose that chronic CBZ exposure of zebrafish males lowered plasma androgens via reduced production in testes by interfering with enzymes involved in steroidogenic pathway as indicated in rats (de Oliva & Miraglia, 2009; Kühn Velten et al., 1990).

4.5 CONCLUSIONS

This study provided evidence of direct effects of gemfibrozil and carbamazepine on steroid production in zebrafish testes. GEM may act directly by modifying plasma lipid balance and limiting the availability of cholesterol, or indirectly through reduced transcription/translation/phosphorylation of StAR, or by interfering with cell signaling cascades. However, the specific intercellular targets of GEM remained unclear. A better understanding of the interaction of GEM with fish PPARs in vivo, and the specific role of PPAR subtypes in fish testes are needed to decipher the mode of action of this environmentally relevant pharmaceutical residue. CBZ altered steroid production by acting at a step distal to the synthesis of pregnenolone from cholesterol. However, the specific genes and/or enzymes being affected need to be identified. Transcriptomics of testes and/or testing of the direct effects of CBZ on the activity of steroidogenic enzymes in testicular microsomes could provide better insights. The current study was a step towards determining the mechanism of action of adverse effects of CBZ and GEM in fish and suggested that in vivo reductions of 11KT are likely due to effects of the pharmaceuticals at the testes and a reduction of steroidogenesis.

4.6 FIGURES

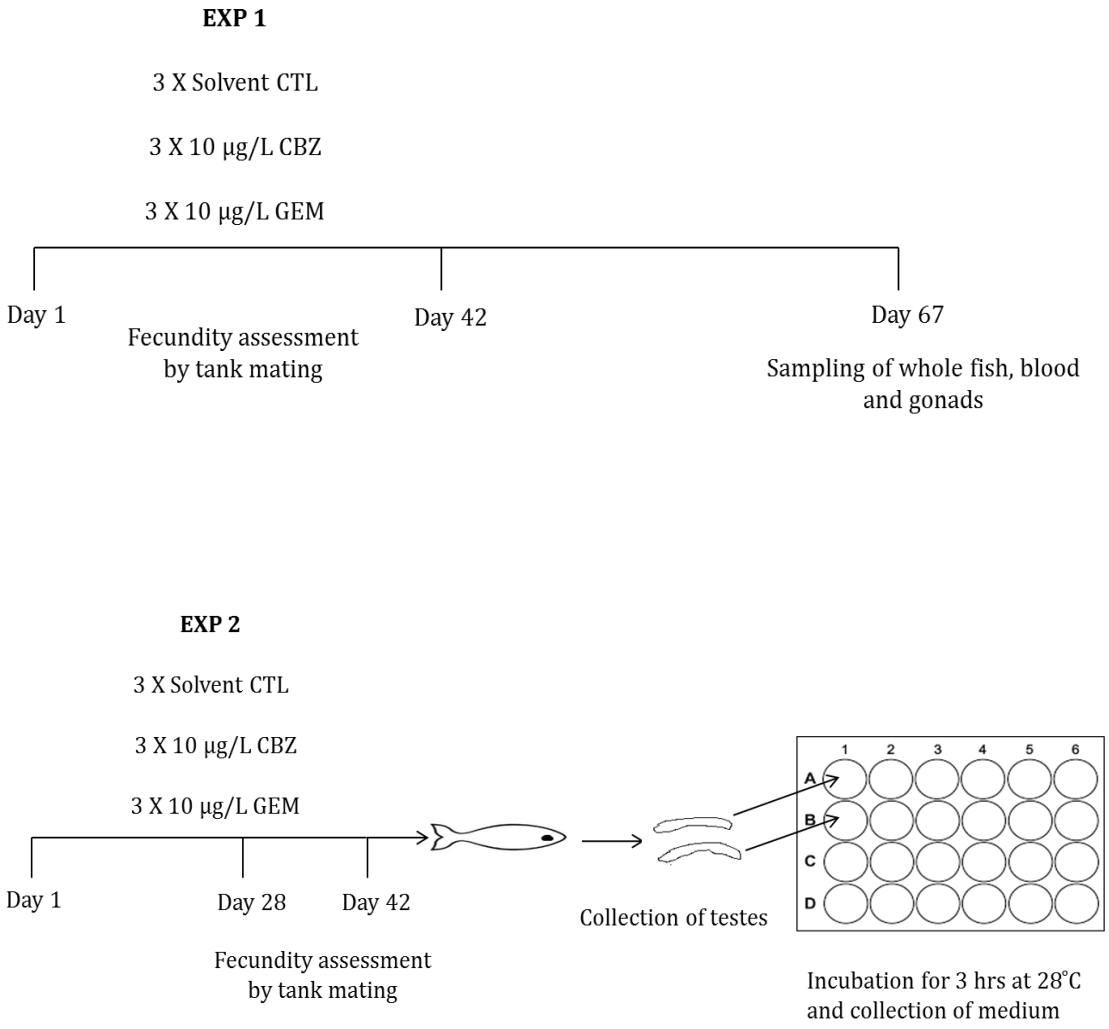

Figure 4.1: Schematic of experiments for assessment of reproductive output and 11-ketotestosterone levels in vivo (Exp. 1) and pre-exposure for ex vivo testes tissue culture (Exp. 2). In Exp. 1, whole tank fecundity was determined daily from day 1–42. After 42 days, non-invasive testing of the exposed animals was conducted until day 67 when the experiment was terminated and plasma, gonad, and whole bodies were sampled for steroid analyses. The exposure was maintained throughout. In Exp. 2, whole tank fecundity was determined from day 28–42 only and after 42 days the testes were removed for ex vivo culture. The exposure was maintained throughout.

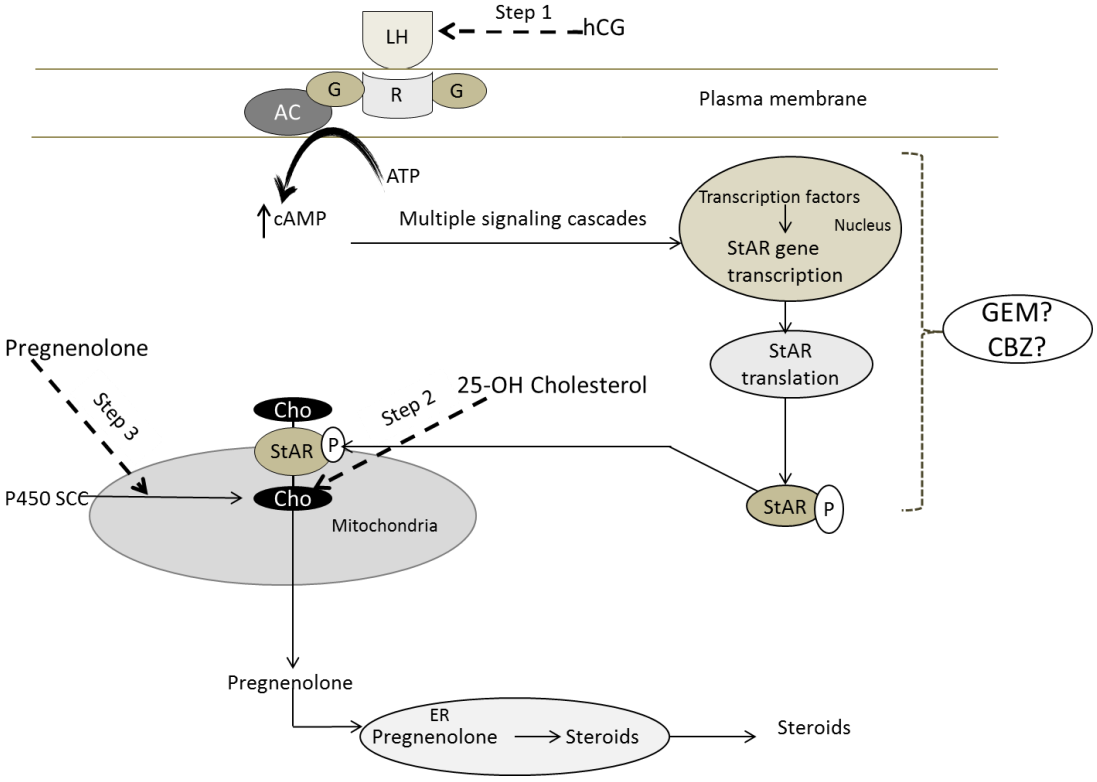

Figure 4.2: Simplified steroidogenic signaling pathway (Adapted from Stocco & Clark 1996; Cameron, 2011). Putative site of action of GEM and CBZ are identified. Broken arrows indicate the steps probed to study the effects of CBZ and GEM on production of 11-ketotestosterone ex vivo. Testis explants were removed from fish after pre exposure to GEM and CBZ and effects of step specific stimulants i.e. human chorionic gonadotropin, 25-OH Cholesterol and pregnenolone were testes by ex vivo incubation. One testis out of the pair was stimulated and the other served as a basal control. [LH–Luteotropic hormone; G–G-protein; R–receptor; AC–adenylyl cyclase; ATP–Adenosine triphosphate; cAMP–cyclic adenosine monophosphate; StAR–steroidogenic acute regulatory protein; GEM–gemfibrozil; CBZ–carbamazepine; P–phosphorylated; Cho–cholesterol; P450SCC–cytochrome P450-side chain cleavage; ER–endoplasmic reticulum].

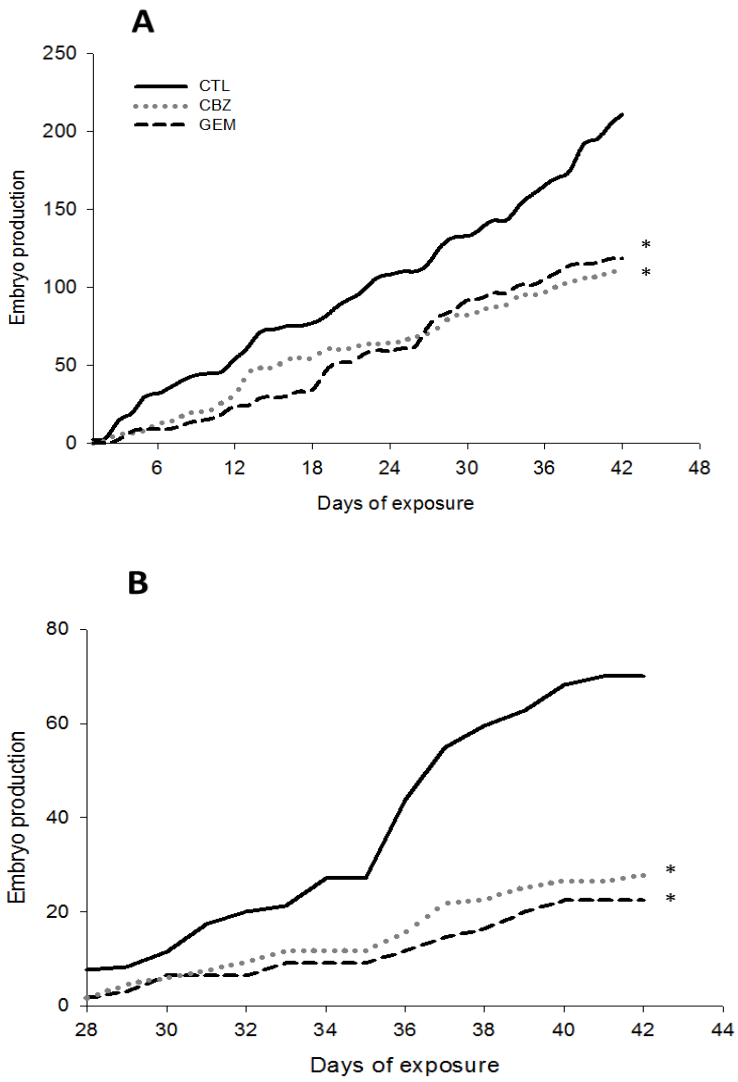

Figure 4.3: Reproductive output determined by whole tank mating. Reproductive output from control, 10 µg/L carbamazepine (CBZ), and 10 µg/L gemfibrozil (GEM) treatment groups are shown as the embryo production normalized per female over time of exposure. Panel A shows the reproductive output from Exp. 1 in breeding assessments carried out from day 1–42 of exposure. Panel B shows the reproductive output from Exp. 2 in breeding assessments carried out from day 28–42 of exposure, immediately prior to testes tissue culture. Asterisks indicate significant differences from the controls; Kruskal-Wallis ANOVA with Dunn's test, $p \leq 0.05$, ($n = 3$ tanks per treatment).

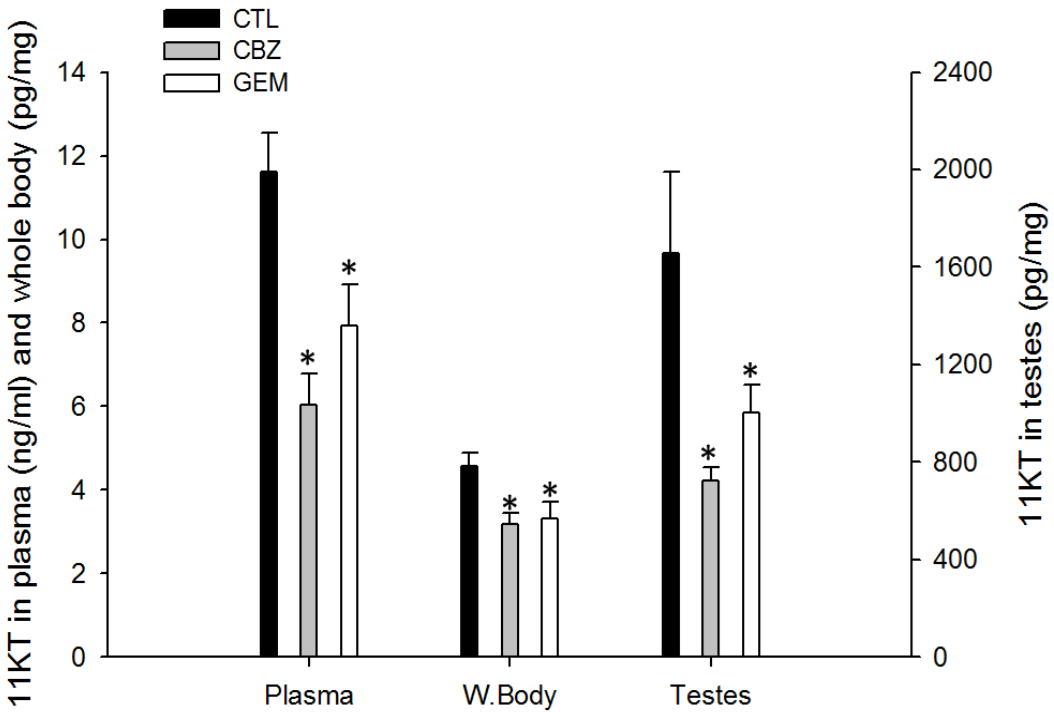

Figure 4.4: Levels of 11 ketotestosterone (mean ± SEM) measured in plasma, whole body, and testes of fish exposed to 10 µg/L of gemfibrozil (GEM) or carbamazepine (CBZ). Asterisks indicate significant differences from the controls, ANOVA with Holm Sidak test; $p \leq 0.05$, (n = 24 – 27 for plasma; n = 15 – 20 for whole body, and n = 9 for testes in each of the three groups).

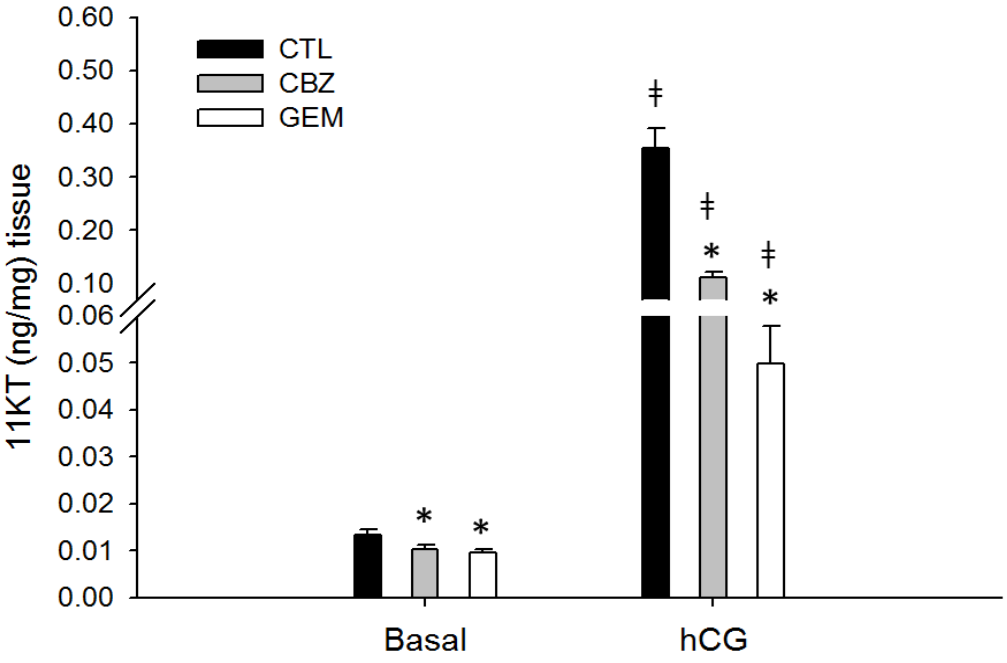

Figure 4.5: Basal and human chorionic gonadotropin (hCG) stimulated 11-ketotestosterone production (mean ± SEM) of testis explants from zebrafish pre-exposed to control (CTL), gemfibrozil (GEM), or carbamazepine (CBZ). Testes were from zebrafish chronically exposed CTL (0 µg/L), CBZ (10 µg/L) or GEM (10 µg/L). Asterisks indicate significant differences between the two exposed groups versus control at either basal or stimulated conditions, ANOVA with Holm-Sidak test, $p \leq 0.05$; while daggers show significant differences between the basal and stimulated response within each treatment (CTL, CBZ and GEM), paired t-test; $p \leq 0.025$, (n = 14–16 fish in each group).

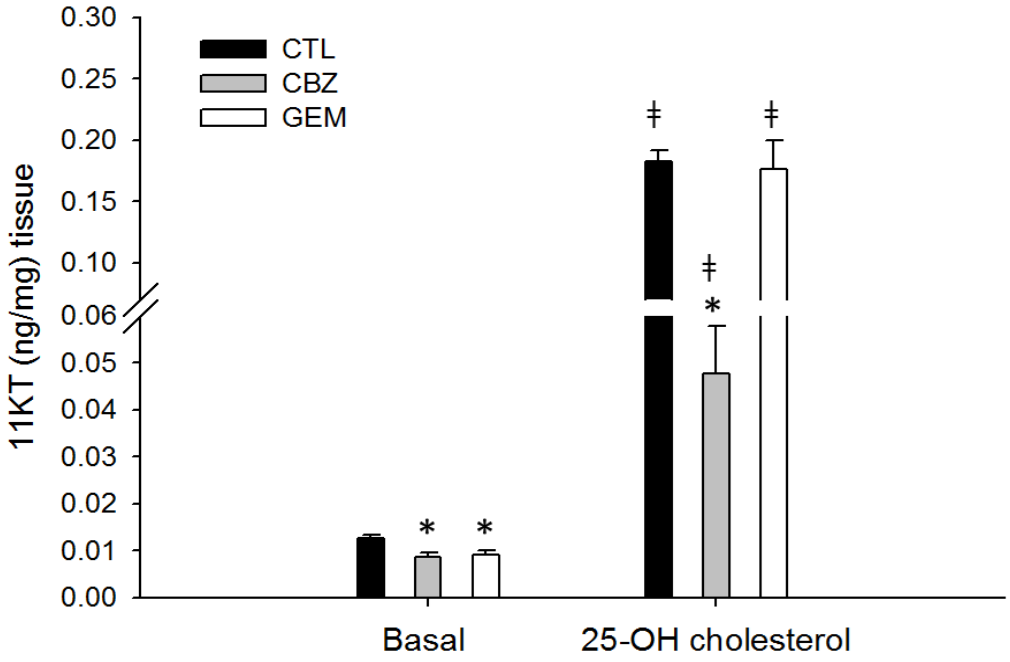

Figure 4.6: Basal and 25-hydorxy cholesterol stimulated 11-ketotestosterone production (mean ± SEM) of testis explants from zebrafish pre-exposed to (CTL), gemfibrozil (GEM), or carbamazepine (CBZ). Testes were from zebrafish chronically exposed CTL (0 µg/L), CBZ (10 µg/L) or GEM (10 µg/L). Asterisks indicate significant differences between the two exposed groups versus control at either basal or stimulated conditions, ANOVA with Holm-Sidak test, $p \leq 0.05$; while daggers show significant differences between the basal and stimulated response within each treatment (CTL, CBZ and GEM), paired t-test; $p \leq 0.025$, (n = 14–16 fish in each group).

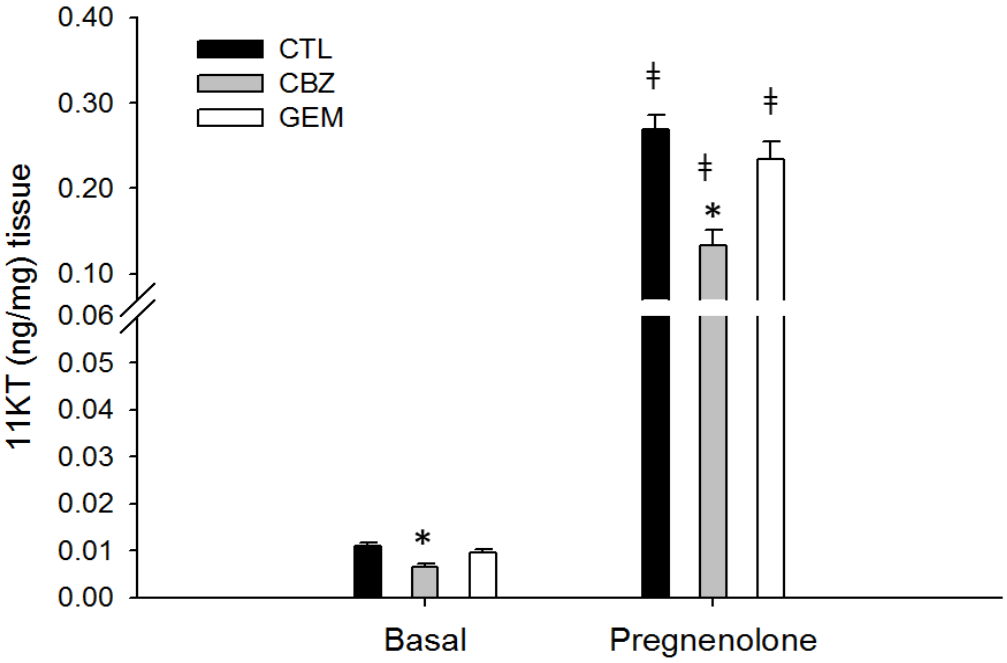

Figure 4.7: Basal and pregnenolone stimulated 11-ketotestosterone production (mean ± SEM) of testis explants from zebrafish pre-exposed to control (CTL), gemfibrozil (GEM), or carbamazepine (CBZ). Testes are from zebrafish chronically exposed CTL (0 µg/L), CBZ (10 µg/L) or GEM (10 µg/L). Asterisks indicate significant differences between the two exposed groups versus control at either basal or stimulated conditions, ANOVA with Holm-Sidak test, $p \leq 0.05$; while daggers show significant differences between the basal and stimulated response within each treatment (CTL, CBZ and GEM), paired t-test; $p \leq 0.025$, (n = 14–16 fish in each group).

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Chapter 5

GENERAL DISCUSSION

This study aimed to examine the effects of chronic carbamazepine (Chapter 2, 4) and gemfibrozil (Chapter 3, 4) exposures on zebrafish reproduction. The experiments were designed to test the hypotheses that parental exposures could negatively impact reproduction in unexposed offspring for multiple generations and assess whether maternal or paternal exposures were relevant for offspring effects (Chapter 2, 3). Lastly, experiments assessed the plausibility of suppressed 11-ketotestosterone production in the testes of males chronically pre-exposed to these compounds (Chapter 4).

5.1 OVERVIEW OF MAIN FINDINGS

5.1.1 Carbamazepine (Chapter 2, Chapter 4)

Exposure of reproductively mature zebrafish for 6 weeks to 10 µg/L CBZ decreased reproductive output as determined through whole tank (Chapter 4) and pairwise mating (Chapter 2). Reduced reproductive output and breeding success occurred in pairwise crosses where exposed males were bred with either control or exposed females, indicating male derived effects (Chapter 2) as suggested by others (Gauls et al., 2013a). This research identified a range of male reproductive measures that were negatively impacted with chronic exposure to CBZ; male courtship and aggression were decreased, sperm presented morphological changes (Chapter 2), and circulating, testicular and whole body 11-ketoteosterone was significantly decreased compared to the respective controls (Chapter 4).

CBZ reduced the production of 11KT in the testes of exposed males, as determined by ex vivo tissue culture (Chapter 4). CBZ pre-exposure caused a suppressed steroidogenic response to hCG stimulation, which could not be restored with 25-OH cholesterol and pregnenolone. In the steroidogenic pathway, CBZ was implied to inhibit a step distal to the production of pregnenolone. Lower production of 11KT in testes of exposed males could explain the significant declines in plasma and whole body in directly exposed F_0 parents; and could be taken as a plausible link between the various endpoints impacted.

Transgenerational paternal effects on fish reproduction were noted in the offspring with CBZ paternal exposure (CBZM/CTLF) because of persistence up to F_3 generation (Chapter 2). Reproductive output was suppressed in F_1–F_3 generations, with altered male courtship behaviour and lower aggression. The sperm of the F_1–F_3 males were morphologically different and swam slower at 20 sec post activation. F_1–F_2 males had lower whole body 11KT than the corresponding controls.

"Single generation-intergenerational effects" (Plaistow et al., 2006) were noted in the lineage with both parents exposed (CBZM/CBZF) as changes were observed in the F_1 generation only (Chapter 2). Lower reproductive output with changes in whole body 11KT, male courtship and sperm morphology was noted in F_1. The range and magnitude of changes in male courtship and sperm morphology decreased gradually in F_2 and F_3. Only male aggression was reduced in F_1–F_3, thus, it was difficult to rationalize transgenerational effects in this lineage, because the effects were not maintained beyond F_1 except for aggression.

5.1.2 Gemfibrozil (Chapter 3, Chapter 4)

Six weeks exposure of adult zebrafish to 10 µg/L GEM produced a decline in fecundity in exposed tanks (Chapter 4). A similar effect with lower breeding success was evident through pairwise crosses in matings including exposed males and females (Chapter 3). Frequencies and durations of male courtship displays were decreased in crosses including GEM exposed males; exposed males showed decreased freezing in front of mirror image relative to the control males. Sperm with longer tails and mid piece (Chapter 3) were found in GEM exposed males. Reduced 11KT was found in plasma, whole body and testes of exposed males (Chapter 4). Testes explants from GEM pre exposed fish had diminished steroidogenic response to hCG, though it became comparable to controls upon stimulation with 25-OH cholesterol and pregnenolone (Chapter 4). This suggests that GEM may interfere with cholesterol transport into the mitochondria (Cameron, 2011) but that HPG signaling is likely unaffected by GEM exposure.

"Single generation-intergenerational effects" (Plaistow et al., 2006) of exposure were seen in the F_1 offspring of the lineage GEMM/CTLF (Chapter 3). Reproductive output had a declining trend in F_1, with reduced male courtship, morphological changes in the sperm and reduced whole body 11KT. Morphological alterations in sperm, higher sperm swimming velocity, and reduced 11KT were found in F_2 offspring. F_3 offspring presented reduced reproductive output and changes in sperm morphology. Anxiolytic effects were only seen in F_2 offspring of the lineage with F_0 male and female exposed to

GEM (GEMM/GEMF). No other lineage showed differences in endpoints studied compared to controls.

5.2 ECOTOXICOLOGICAL RELEVANCE

Study of fish reproduction in response to water contaminants has ecological, economical and scientific applications. Zebrafish is a widely employed model species to study endocrine disruption and suitable for transgenerational experiments (Scholz & Mayer 2008; Hill et al., 2005; Segner, 2009; Gunnarsson et al., 2008; Overturf et al., 2015; Mcgonnal et al., 2006). Its utility in predictive ecotoxicology is highlighted by the fact that it is one model species accredited by OECD to assess reproductive endocrine disrupting chemicals (OECD, 2008).

The choice of test compounds was in part due to the ubiquity of occurrence/frequent detection in aquatic environments (Fent et al., 2006; Gavrilescu et al., 2015; Hughes et al., 2013). The concentrations used in the current study were close to the maximal detected in surface water effluent (11.56 µg/L for CBZ and 7.78 µg/L for GEM; reported by Loos et al., 2009 and Muñoz et al., 2009) and could be realistic for effluent dominated water bodies. Moreover, the concentrations could be relevant considering highly plausible additive or synergistic mixture effects, as normally cocktails of contaminants occur in aquatic environments which can pose mixture effects if contaminants with similar MOA are present concomitantly (reviewed by Backhaus, 2014). For example in sewage effluents and surface water, gemfibrozil commonly co-occurs with other fibrates like clofibrate, bezafibrate and fenofibrate (Santos et al., 2010;

Loos et al., 2009), and other PPAR agonists like phthalates (Fromme et al., 2002) and perfluorinated compounds (Loos et al., 2009); therefore additive mixture effects might be anticipated. Carbamazepine co-occurrs with atrazine, diuron and triclosan in surface water (Arlos et al., 2015; Loos et al., 2009), and based on the anti-androgenic properties of these compounds (Rohr & McCoy, 2010; Rostkowski et al., 2011) the probability of mixture effects could exist. The combined toxicity of such mixtures could be conceptually estimated by component based mixture models (Backhaus et al., 2014). Nonethless it is difficult to predict the environmental outcome of mixture effects, due to complex interaction between biotic and abiotic factors (reviewed by Backhaus et al., 2014).

Chronic exposures were chosen because aquatic species more likely experience long term or lifelong exposures (Daughton & Ternes, 1999). Chronic assays offer higher adequacy to study sub-lethal toxicity, are generally more sensitive than acute tests (Schmitt et al., 2010; Ankley et al., 2007; Fent et al., 2006), and offer the advantage of studying the sub-lethal adverse effects of EDCs in intact organisms (Ankley et al., 2007; Nichols et al., 2011; Daughton & Ternes, 1999). There is a general lack of chronic toxicity data for pharmaceutical reprocutive endocrine disruptors, with the exemption of ethinyl estradiol (Fent et al., 2006; Overtuf et al., 2015); this thesis research fills an important knowledge gap for the types of exposures most relevant for fish exposed in the wild. The direct exposure of the parental fish in our experiment could be equivalent to a partial life cycle test, since reproductively mature fish were treated with a test chemical in the critical window of active reproduction to see the effects likely through sexual

endocrine disruption (Ankley & Johnson, 2004). Multigenerational effects monitoring in the unexposed offspring was considered since EDCs can lead to delayed effects in the offspring in the absence of exposure and modified phenotypes may persist in future generations (Head et al., 2012; Casati et al., 2015; Schwindt, 2015). It has been suggested that negatively altered phenotypes, which persist for several generations, may have the potential to affect population health (Head et al., 2012; Head, 2014; Vandegehuchte & Janssen, 2011; 2014).

Integrative ecotoxicological studies investigate the effects of contaminants at various levels of biological organization; i.e. molecular/cellular, organ/tissue, individual, community and population level responses, such that mechanistic understanding is maximal at the molecular level and ecological relevance at the population level (Ankley et al., 2010). The endpoints evaluated in the current study are environmentally suitable indicators of endocrine pathways in fish (reviewed by Manibusan & Touart, 2017). Fecundity (cumulative, mean embryo production), breeding success, sex ratios and viability and survival of embryos, are common endpoints included in partial life cycle tests and can contribute significantly to population level effects (Archand-Hoy & Bensen, 1998; Ankley & Johnson, 2004; Hutchinson et al., 2006; OECD, 2008). Sperm quality (morphology and velocity), circulating 11-ketotestosterone, sexual behaviour and male aggression are individual level measures of male reproductive performance; whereas the behavioural changes can effect individual competitive reproductive fitness and potentially have direct or indirect ecological consequences (Tokarz et al., 2013; Cohn & MacPhail, 1996; Brodin et al., 2014; OECD, 2008). In our study, the reduced production of 11KT in

testes of exposed fish could be taken as an organ level measure, as steroid synthesis pathway could be a target for reproductive EDCs (Manibusan & Touart, 2017).

In conclusion, we saw negative impacts on almost all the male derived reproductive indices studied in response to direct exposure, and it would be reasonable to suggest the androgen lowering potential of carbamazepine and gemfibrozil with negative impacts on male sex hormone associated physiological processes. Our findings integrate with existing fish literature in terms of EDCs exposure outcomes that could be explained by epigenetic mechanisms (reviewed by Schwindt, 2015). However, as with mammals, the potential long-term consequences of transgenerational inheritance in fish on evolutionary consequences remain unknown and rather controversial.

5.3 REPRODUCTIVE ENDOCRINE DISRUPTION

Classical endocrine active contaminants are agonists or antagonists of estrogen and androgen receptors and act by directly interfering with the corresponding receptor signaling pathways. Recently, the concept was broadened to non-receptor mediated endocrine disruption, which describes that endocrine effects could be manifested through pathways independent of pharmacological interactions with sex hormone receptors (Tyler et al., 1998; Söffker & Tyler, 2012; Fisher, 2004). The mechanisms incorporate altered expression of steroid receptors (e.g. dieldrin) and altered binding of transcription factors and co-activator proteins to steroid responsive genes, leading to modified gene expression (e.g. BPA; Martyniuk et al., 2010; Tabb & Blumberg 2006). Other modes include the disruption of synthesis, transport or metabolism of endogenous sex hormones, such as, by

inhibition of ovarian aromatase (e.g. percholarz), alteration of cholesterol availability or activity of P450-SCC (e.g. β-sitosterol), inhibition of 3-β HSD (e.g. trilostane), and increasing metabolic clearance (e.g. fenarimol; Overturf et al., 2015; MacLatchy et al., 1995; Villeneuve et al., 2008; Thibaut et al., 2004). Still there are non-steroidal anti-inflammatory drugs like ibuprofen and indomethacin, which are suggested to disrupt fish reproduction by impeding the production of prostaglandins through COX inhibition (Han et al., 2010; Lister & Van Der Kraak, 2008).

The test compounds utilized in this thesis (CBZ and GEM) might be proposed as endocrine active pharmaceuticals, since we observed diminished basal steroidogenic production and upon hCG stimulation in pre exposed testes explants (Chapter 4). For CBZ, we confirmed the hypothesis of reduced testicular production of 11KT however; other mechanisms of reduced circulating steroids have been proposed in mammals (Macphee et al., 1988; Isojärvi et al., 1995; Bauer et al., 2004). We were not able to test the hypothesis of induction of sex hormone binding globulin (SHBG) synthesis for lowering the circulating hormone (Isojärvi et al., 1995) due to time constraints, and thus cannot rule this out as contributing to lower plasma 11KT levels in zebrafish after CBZ exposure in vivo (Chapter 4). Regardless of poor coding sequence similiarity between the human and fish SHBG, the steroid binding sites are highly conserved (Miguel-Queralt et al., 2004); hence further research should be directed towards understanding the role of SHBG with respect to CBZ expsoure.

GEM is accepted to mediate pharmacological actions though activation of PPARα in mammals. PPAR is included in the list of pathways pertinent to study endocrine disruption, since it can perturb steroidogenesis and the physiological processes regulated by sex hormones (reviewed by Manibusan & Touart, 2017). Activation of PPARα by environmental chemicals can affect male mammals by impairing the rate limiting step of cholesterol availability, for the synthesis of testosterone in the testes (reviewed by Manibusan & Touart, 2017). The assumption of an analogous mechanism in fish might be proposed by reports in gold fish and zebrafish males, as both express PPARs in testes (Ibabe et al., 2002; Cameron, 2011). Lower testosterone production due to impaired StAR mediated cholesterol transport with gemfibrozil and fenofibrate treatment was seen in gold fish (Cameron, 2011); and we found lower 11KT production in zebrafish testes in response to GEM exposure (Chapter 4). Broadly, there is a poor understanding of function of fish PPARs regardless of the fact that various fish including zebrafish express all three PPARs i.e. PPARα, PPARβ, and PPARγ (Ruyter et al, 1997; Leaver et al., 1998; Mimeault et al., 2006; Ibabe et al., 2002). It could be implied from the available fish studies, that it is unclear if fibrates mediate effects through interaction with PPARα or through non selective interactions with other PPAR subtypes (Mimeault et al., 2006; Velasco-Santamaría et al., 2011; Skolness et al., 2012; Prindiville et al., 2011). Fibrates including GEM failed to alter the expression of PPARα mRNA in fish liver and caused weak or no changes in indicators of peroxisomal proliferation response (Mimeault et al., 2006; Scarano et al., 1994; Weston et al., 2009; Skolness et al., 2012; Prindiville et al., 2011). However, PPARβ transcripts were altered in goldfish liver with GEM exposure

(Mimeault et al., 2006) and in zebrafish testes with bezafibrate exposure (Velasco-Santamaría et al., 2011). Upregulation of PPARα in liver and PPARβ in ovaries of zebrafish after exposure to clofibric acid was reported (Coimbra et al., 2015). Thus, the direct evidence of specific subtypes of PPARs mediating the effects of fibrates in fish is yet unclear.

5.4 NEUROENDOCRINE DISRUPTION

Behaviours are responses which present outcomes of complex underlying physiological interactions among various endocrine axes and neuronal pathways (as depicted in Fig. 1, by Filby et al., 2010). There exists a complex interplay between gonads, hormones and endocrine activity of brain, which is integrated by the concept of neuroendocrine disruption. Environmental contaminants can cause neuroendocrine disruption via pharmacological interactions with neuropeptides, neurotransmitters, or neuro-hormones, or altering their turnover rates (Waye & Trudeau, 2011). The resultant effects could manifest as alterations in an individuals ability to handle stress, associate socially, or reproduce successfully (Waye & Trudeau, 2011). For example, EE_2 upregulated, while fadrozole downregulated, the expression of CYP19b in hypothalamus and telencephalon of goldfish (Martyniuk et al., 2010; Zhang et al., 2009) and fluoxetine (selective serotonin reuptake inhibitor) disrupted fish reproduction by reducing isotocin in the telencephalon of goldfish (Menningen et al., 2010). Thus, special consideration should be given to the behavioural alterations observed in response to exposure to our test compounds, because these might be related to the neuroendocrine effects of CBZ and GEM.

The hypothesis of neuroendocrine effects may be of greater importance with respect to CBZ, which is a lipophilic, neuroactive compound with multiple pharmacological modes of action and is able to cross the blood brain barrier in fish (Garcia et al., 2012; Kulkarni et al., 2014). CBZ alters neurotransmitters in the mammalian brain and increases the balance of brain dopamine and extracellular serotonin in the hippocampus (reviewed by Ambrosio et al., 2002). Such effects of CBZ in fish have not been assessed but might be considered relevant. In several teleost fish species including zebrafish, dopamine is a gonadotropin releasing hormone (GnRH) inhibitory factor that inhibits the basal and GnRH stimulated release of LH, reduces the expression of and decreases signaling through GnRH (Dufour et al., 2005; Fontaine et al., 2013). In goldfish, dopamine interferes with the interaction between pheromones and the HPG axis and modulates spawning behaviour (Dulka et al., 1992). Arginine vasotocin (AVT) is a neuropeptide involved in social behaviour and aggression in fish and its transcription is regulated by dopamine and serotonin (Menningen et al., 2011; Popesku et al., 2008). In zebrafish and other teleost species, brain serotonin is related to social status, such that high serotonin is linked with lower aggression or timid behaviour (reviewed by Lillesaar, 2011; Fiby et al., 2010); and this might explain the plausible basis of reduced aggression in CBZ exposed males in our study. In summary, the above studies point towards the need of much broader mechanistic understanding of the neuroendocrine effects of CBZ, similar to the exemplary studies completed with fluoxetine (reviewed by Menningen et al., 2011).

As opposed to the strong links between neuroendocrine function and the mode of action of CBZ, it appears difficult to comprehend or link neuroendocrine physiology with gemfibrozil actions due to complex physiological roles of PPARs. Nonetheless, the PPAR pathway is included in the list of pertinent assay and endpoints for studying behaviour in OECD 21 day fish toxicity and fish life cycle tests (reviewed by Manibusan & Touart, 2017). Signaling cross talk between PPAR and ER is reported in rats (Lemberger et al., 1996). And a PPARα-RXR heterodimer response element was detected in the promoter region of zebrafish brain aromatase (CYP19b; Kazeto et al., 2011). CYP19b is highly expressed in radial gilal cells of fish brain and is quite inducible by estrogens and aromatizable androgens (Diotel et al., 2011). Brain aromatase plays an important role in sexual behaviour and aggression in fish (Diotel et al., 2011). Another mechanistic clue might be extended from the implied links between cholesterol, anxiety/depression, serotonin, and the HPA axis as suggested in humans (Papakostas et al., 2004). However, the exact links are not fully understood in mammals and the direct testing of these hypotheses with GEM treatment in fish is nonexistent to our knowledge.

One aspect of developmental physiology that remained under investigated in our experiments was the possibility of the test compounds to impact zebrafish sex determination and differentiation (at least for the F_2 and F_3 offspring; for F_1 see Hammill et al., submitted). Zebrafish sex determination is complex and poorly understood and has a polygenic control that responds to environmental influences (Orban et al., 2009; Liew & Orban, 2014). We should acknowledge that CBZ and GEM might interfere with sex determination at early stages of development in two ways; a) exposure of embryo from

maternal deposition (a possibility which might have existed in F_1 only; Ostrach et al., 2008; Van der Kraak et al., 2001), and b) altered deposition of hormones/proteins/RNAs in the embryo from the gametes (a possibility which might be more plausible in unexposed F_2 and F_3 offspring; Devlin & Nagahama, 2002; Bobe & Labbé, 2010). Fish early life stages could be considered sensitive to EDCs expsoure (Van der Kraak et al., 2001), since the brain and the HPG axis are organized early in development in a sexually dimorphic manner, and HPG is activated later by the gonadal steroids (Walker & Gore, 2014). Sex steroid production and gonadal differentiation are two closely inter-related events in development (Devlin & Nagahama, 2002). The brain and gonads have a complex interaction to affect multiple biochemical, physiological and neurological processes to control the gonadal differentiation (Devlin & Nagahama, 2002). Although CBZ and GEM have been shown to bioconcentrate in various fish species (Ramirez et al., 2007; Garcia et al., 2012; Valdés et al., 2014; Mimeault et al., 2005); the concentartions of CBZ and GEM in the eggs from directly exposed F_0 in our experiments could not be measured because of a lack of appropriate methods to extract these compounds from yolk. We have not studied the transcriptome of the developing offspring brain/gonads, or carried out histological examination of the gonads during sex differentiation, or in adulthood (except for the F_1, Hammill et al., submitted). However, we dissected 350 to 400 reproductively mature adult fish per generation and did not find un-differentiated gonads (visual observations) in the offspring, or changes in secondary sex characteristics in the offspring (i.e. secondary characteristics matched gonads). The work of Hammill et al., (submitted) examined the gonadal histology of the F_1 offspring from our experiments

and confirmed our visual findings of male biased sex ratios in F_1 adults through gonadal development, in one lineage with CBZ paternal expsoure (CBZM/CTLF) and two lineages of GEM expsoure background (GEMM/CTLF and GEMM/GEMF). The biased sex ratios were found to persist in the F_2 offspring of the aforementioned lineages though visual inspection of secondary sex characters at sexual maturity (data not shown). Nonetheless, to get a mechanistic understanding of such findings, it would be intriguing to look at the expression of some candidate genes involved in sex differentiation, like CYP19a (gonadal aromatase), CYP19b (brain aromatase), sox-9 (Sry-related HMG box gene 9), amh (anti-Müllerian hormone), and foxl2 (forkhead box protein L2, Pardhan et al., 2012); and signaling pathways involved in sex differentiation like Tp53-apoptosis (tumor protein 53), NF-ƙB ((nuclear factor of kappa light polypeptide gene enhancer in B cells) and canonical Wnt (Wnt/beta-catenin, Liew & Orban, 2014).

5.5 MULTI/CROSS-GENERATIONAL EFFECTS & TRANSGENERATIONAL INHERITANCE

A broad distinction between transgenerational and multigenerational effects could be that the former are noticeable in the absence of the initial triggering stimulus (diet, environment, chemical exposure, stress etc.) whereas the later effects are "context/experience dependent" and prevail in the presence of the stimulus (Bruggen & Crews, 2014). Both types may incorporate epigenetic changes and have similar classification (paternal, maternal, and parental effects), but diverge from the aspect of context endurance (Bruggen & Crews, 2014). With exclusive male parent exposure, effects into the F_2 generation could be considered as transgenerational effects since F_1

germline is exposed (Aiken & Ozanne, 2014; Bruggen & Crews, 2014). With both parents exposed, maternal deposition in fish and placental contact in mammals can expose F_1 offspring with F_2 germline, and thus only the effects in F_3 could be free of the context of exposure and called as transgenerational effects (Nilsson & Skinner 2015; Youngson & Whitelaw, 2008; Bruggen & Crews, 2014). Transgenerational effects can be inherited by germline through various routes; a) triggering of the epigenetic change in the germline, b) comparable stimulation in somatic and germline cells, and c) triggering of the epigenetic change in somatic cells and transmission to germline cells (Jablonka & Raz, 2009).

With F_0 CBZ exposure, we suggest transgenerational paternal effects in the CBZM/CTLF lineage because of inclusion of exposed F_0 male to generate this lineage and persistence of effects in almost all the endpoints under examination up to the F_3 generation of unexposed male offspring (Chapter 2). Paternal transgenerational effects are explained by germline inheritance and imprinting of genes, so that the "acquired epigenetic memory" could be maintained across multiple generations and can impart an identicalness between parent and offspring (Curley et al., 2011; Chen et al., 2016; Danchin et al., 2011); as might be implied from our behavioural data (Chapter 2). The presumed underlying explanation of paternal epigenomic inheritance aligns with recent reports that show a dominant role of the zebrafish sperm epi-methylome in early embryos, stable inheritance and maintenance of sperm DNA methylome (Jiang et al., 2013; Potok et al., 2013). Paternal epigenetic inheritance is not a phenomenon limited to zebrafish, and despite the controversy of widespread demethylation, in germline and zygote

development, such effects are reported in mammals (as reviewed by Aiken & Ozanne, 2014; Schwindt, 2015) and are clearly supported by IVF experiments where only sperm heads were injected into the eggs (reviewed by Chen et al., 2016). However, both in zebrafish and mammals, the exclusive role of the altered sperm DNA methylome in maintaining stably inherited phenotypes across generations is considered unclear (Chen et al., 2016; Hacket & Surani, 2013). The exact mechanisms of paternal epigenetic inheritance are not known, however, sperm RNAs are suggested as the transmitters of acquired epigenetic paternal inheritance through "RNA-guided DNA methylation" control (Chen et al., 2016; Hacket & Surani, 2013). The role of sperm miRNAs and tsRNAs in transmitting the paternal epigenetic effects has been clearly shown in rodents (reviewed by Chen et al., 2016).

"Context dependent" multigenerational effects limited to one generation could be termed as "single generation-intergenerational effects" (Bruggen & Crews, 2014; Plaistow et al., 2006). These kinds of effects were implied in a second lineage (CBZM/CBZF) from F_0 CBZ exposure, because the effects were limited to the F_1 generation (Chapter 2). Despite the exposure of both male and female F_0 parents, the effects noted in F_1 don't essentially lead to the interpretation of balanced contributions of sperm and egg; rather express the indistinct role of both parents (Schwindt, 2015). However, the presumable paternal effects could be indirectly inferred from our data in this lineage (CBZM/CBZF), because of the absence of effects in the lineage with F_0 maternal exposure history (CTLM/CBZF, Chapter 2). Finally, "single generation-intergenerational" effects (Plaistow et al., 2006) of GEM exposure were suggested in F_1

offspring of GEMM/CTLF lineage, because effects did not last beyond F_1 generation and the lineage was composed with exposed F_0 males in crosses (Chapter 3).

The theoretical models for transgenerational effects "wash out" were reviewed by Bruggen (2015). Practically very few animal studies have pursued such effects beyond F_3 generation. Nonetheless, a gradual decline of the observed effects from F_1 to F_3 generations was suggested with support from studies in rodents (Bruggen, 2015). Our findings conform to this proposition as well. "Active" abatement could occur due to decrease in epigenetic marks as patterns of DNA methylation, histone and chromatin modifications vary from one generation to another (Bruggen, 2015).

5.6 FUTURE DIRECTIONS

This research raised many interesting questions that require future studies, some of which I have outlined below. The impacts of CBZ on behaviour, coupled with the fact that CBZ is a neuroactive compound in humans, strongly points to the need for an, investigation into possible modes of action of CBZ on the neuroendocrine system fish. It is very important to understand if CBZ exposure leads to a rise in serotonin in fish neuroendocrine regions and whether brain dopamine concentrations are altered. Such investigations could be rationalized due to the importance of these neurotransmitters in signaling of the HPG axis and behaviour in fish and the link previously outlined between CBZ exposure and altered serotonin and dopamine in mammals. This research focus could direct the study of potential interactions of CBZ with LH release. As this thesis

suggested paternal transgenerational effects, it would be meaningful to study the role of CBZ to alter epigenome in fish sperm and its ability to affect the quality of fish gametes.

It seems very important to better understand the function of fish PPARs in lipid metabolism, especially with respect to PPARα and reproduction, because a whole range of environmentally relevant contaminants like phthalates, plasticizers, fluorinated compounds, and fibrates interact with these receptors in mammals. Equally important is to examine if GEM interacts selectively with PPARα or non-selectively with different subtypes of PPARs in fish. A better understanding of the physiological roles of PPARs in fish reproduction is needed to comprehend the similarities and differences in PPAR function across vertebrates, which is a major limitation for extrapolating data from mammalian fibrate exposure studies.

This study indicated that GEM may act directly by modifying plasma lipid balance and limiting the availability of cholesterol. However, the specific intercellular targets of GEM need to be examined, which likely includes the signaling cascades regulating the synthesis and/or phosphorylation of StAR. Moreover, we suggested that CBZ might to be altering steroid production by acting at a step distal to the formation of pregnenolone from cholesterol (the common steroidogenic precursor). With regard to CBZ, CYP17a, CYP11b, HSD-17β3 and HSD-11β2 might be proposed as presumable targets for future investigations, since CYP17a catalyzes a step that can holdup the synthesis of sex steroids, while CYP11b is involved in formation of 11KT precursors and HSD-17β3 and HSD-11β2 at the final steps of 11KT synthesis.

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