

Embryotoxicity of fluoxetine (Prozac[®]) to Japanese medaka
(Oryzias latipes)

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ABSTRACT

Fluoxetine hydrochloride (FLX), Prozac[®], is a selective serotonin reuptake inhibitor (SSRI) commonly prescribed to patients suffering from depression and other psychiatric disorders. FLX is known to enter the aquatic environment through municipal effluent discharge so that the constant release of this drug into aquatic environments exposes all organisms throughout all stages of development. In the current study, we evaluated the role of FLX in aquatic toxicology by modeling the toxicity of maternally-transferred compounds on fish embryo development using Japanese medaka (*Oryzias latipes*) as a model species. Three different methods: (1) waterborne exposure, (2) nano-injections and (3) topical application were considered and undertaken to measure the embryotoxicity of FLX. Waterborne exposure and nano-injection presented difficulties in mimicking the transfer of contaminated maternal tissue to embryos; thus, topical application was used as the method of choice. Dosing for toxicity tests was performed by topical application of FLX in dimethylsulfoxide (DMSO) onto the exterior of newly fertilized eggs to transport the chemical into the egg. Toxicity was assessed by observing how it affected mortality and adverse fish development, as indicated by the frequency of signs of pathology in fish embryos. From the results, FLX does cause developmental abnormalities as observed from the significant decrease in the percentage of normal fish. This occurrence is mostly contributed from a high prevalence of body hemorrhaging in comparison to the other signs of pathology. Thus, FLX may be maternally-transferred to oocytes during sexual maturation to cause embryotoxicity.

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TABLE OF CONTENTS

Title Page.....	1
Abstract.....	2
Acknowledgements.....	3
Table of Contents.....	5
List of Abbreviations.....	7
List of Figures.....	8
List of Tables.....	9
Introduction and Literature Review.....	10
• Profile on Fluoxetine (Prozac [®]).....	10
• Fluoxetine in the Aquatic Environment.....	11
• Mechanism of Toxicity.....	14
• Rationale and Study Objective.....	16
Materials and Methods.....	18
• Experimental Design.....	18
• Medaka Egg Production.....	19
• Standard Bioassay Procedures.....	19
• Method 2: Nano-Injection.....	25
• Method 3: Topical Application.....	28
Results.....	30
• One-hour fluoxetine exposure.....	30
• Thirty-minute fluoxetine exposure.....	32

• Prevalence of signs of pathology and other observations for 30 minute exposure.....	34
• Table 2: Toxicity of fluoxetine to Japanese medaka.....	39
Discussion.....	40
• Limitations of experimental design.....	41
• Abnormal signs of pathology.....	44
• Decreased percent normal from fluoxetine exposure.....	46
• Environmental significance.....	49
• Conclusion.....	50
Literature cited.....	51
Summary.....	57
Appendices.....	58

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
BCF	Bioconcentration Factor
CYP	Cytochrome P450
CYP1A	Cytochrome P450 1A
dpf	days post fertilization
DMSO	Dimethyl Sulfoxide
ERS	Embryo Rearing Solution
FLX	Fluoxetine Hydrochloride
GnRH	Gonadotropin Releasing Hormone
K_{ow}	Octanol-Water Partition Coefficient
MELA	Medaka Embryo-Larval Assay
NFLX	Norfluoxetine Hydrochloride
PEG	Polyethylene glycol
PG	Prostaglandin
rpm	revolutions per minute
SSRI	Selective Serotonin Reuptake Inhibitor
WWTP	Wastewater Treatment Plant

LIST OF FIGURES

Figure 1: Images of medaka embryos taken 12 dpf and after topical application of FLX...	24
Figure 2: Nano-injection of triolein and aqueous solution in medaka egg.....	26
Figure 3: One-hour fluoxetine exposure.....	31
Figure 4: Thirty-minute fluoxetine exposure.....	33
Figure 5: Prevalence of signs of pathology (YSE, PE, AED, FR, SM, and CF) for 30 minute exposure.....	36
Figure 6: Prevalence of body hemorrhaging (BH) for 30 minute exposure.....	37
Figure 7: Prevalence of other signs of pathology (ASYM and EBO) for 30 minute exposure	38
Appendix 1: Chemical structure of fluoxetine hydrochloride and norfluoxetine hydrochloride.....	59
Appendix 2: Mechanism of SSRIs.....	59

LIST OF TABLES

Table 1: Considerations on Methods.....	18
Table 2: Toxicity of Fluoxetine to Japanese medaka.....	39

INTRODUCTION AND LITERATURE REVIEW

Profile on Fluoxetine (Prozac[®])

Fluoxetine (FLX), (\pm)-N-methyl- γ -[4-(trifluoromethyl)phenoxy]-benzenepropanamine hydrochloride, an antidepressant from the class of selective serotonin reuptake inhibitors (SSRIs), was manufactured and introduced into the market under the brand name Prozac[®] almost 20 years ago by Eli Lilly (Metcalf *et al.*, 2010). Since its introduction, FLX (appendix 1) has become one of the most widely prescribed drugs worldwide. Between the years 2000 to 2005, FLX prescription rates in the US have increased from 7.3 million to 23 million, showing an increase in usage by 315% (IMS Health, 2006). This increase can be attributed to the broad spectrum of usage for FLX in the treatment of clinical depression, obsessive-compulsive behaviours, panic disorders, and other psychiatric disorders (Wong *et al.*, 1995; Oakes *et al.*, 2010).

SSRIs, including FLX, act primarily on the central nervous system by affecting concentrations of serotonin (5-hydroxytryptamine [5-HT], a chemical neurotransmitter) (Oakes *et al.*, 2010; Henry *et al.*, 2004). FLX inhibits serotonin re-uptake at the presynaptic neuronal membrane to generate an increase in serotonin levels at the postsynaptic receptor sites and synaptic cleft to allow for transmission by serotonergic neurons (appendix 2) (Oakes *et al.*, 2010; Henry *et al.*, 2004). In addition to serotonin acting as a neurotransmitter in the brain, it may also affect the immune system, behaviour, appetite and alter sexual function (Fent *et al.*, 2006). However, when comparing SSRIs to other psychotropic drugs used for the treatment of clinical depression, FLX and other SSRIs are considered relatively safe due to their low affinity for neurotransmitter receptors, which decreases the incidence of serious side effects (Oakes *et al.*, 2010).

Prozac[®], commonly orally administered, is made from a 50/50 racemic mixture of R- and S-enantiomers in which both stereoisomers can be completely absorbed by the human body (Hiemke and Härter, 2000). The enantiomers from this chiral compound have been known to differ in their effects on organisms. The S-enantiomer of FLX was much more toxic than the R-enantiomer (Stanley and Brooks, 2009; Hiemke and Härter, 2000). Assessment of the survival and growth of fathead minnow (*Pimephales promelas*) from Stanley *et al.* (2007) found that the S-enantiomer was 1.6 and 3.3 times more potent than the R-enantiomer, respectively.

In the human body, FLX is metabolized through the activity of a cytochrome P-450 (CYP) isoenzyme, CYP2D6, into its demethylated metabolite norfluoxetine (NFLX) (Hiemke and Härter, 2000). NFLX (appendix 1) is structurally similar to its parent compound which makes it equipotent to organisms (Paterson and Metcalfe, 2008; Brooks *et al.*, 2005). After human consumption, the main excretion route for FLX is via urine whereby 11% of the ingested dose is excreted as the parent compound and 7% of the dose is excreted as NFLX (de Vane, 2000). This information is important as pharmacokinetics data from human and mammalian models have indicated that FLX and its metabolite, NFLX, has a relatively long half-life of 1 to 6 days and 7 to 16 days respectively, while other SSRIs have half-lives of ≤ 1.5 day (Hiemke and Härter, 2000; Kreke and Dietrich, 2008). Once FLX is excreted from the human body, it is discharged as municipal effluents into the aquatic environment.

Fluoxetine in the Aquatic Environment

Antidepressants pharmaceuticals are becoming more of interest as potential aquatic contaminants due to their presence in water bodies of concern (Kolpin *et al.*, 2002; Brooks

et al., 2005; Vasskog *et al.*, 2006). The quantity of these drugs entering the aquatic environment primarily through municipal effluent discharge is highly dependent on human usage, pharmacokinetic and physiochemical properties of the drugs, and sewage and wastewater treatment processes (Daughton and Ternes, 1999). Over the years, the widespread use of these compounds has led to the contamination of surface water, ground water, and municipal wastewater in North America and northern Europe (Kolpin *et al.*, 2002; Metcalfe *et al.*, 2003; Zorita *et al.*, 2007; Schultz and Furlong, 2008). The constant release of these drugs into aquatic environments also exposes all organisms throughout all stages of development and it has made these contaminants pseudo-persistent in the environment (Daughton, 2002). Pseudo-persistence occurs when there is continual release of the compound into the environment to replenish their supply even though their half-lives may be short. The pseudo-persistence of antidepressants is critical as fish collected from receiving waters near wastewater treatment plants (WWTPs) have accumulated concentrations of antidepressants, FLX and sertraline, in their tissue (Brooks *et al.*, 2005; Chu and Metcalfe, 2007).

Specifically, the presence of FLX in surface waters and municipal effluents has adversely affected exposed aquatic flora and fauna ecosystems as it has a half-life of 2 to 6 days in lake water and sediments (Kolpin *et al.* 2002, Metcalfe *et al.* 2003a, 2003b, Johnson *et al.*, 2005; Kwon and Armbrust, 2006; Lam *et al.*, 2005). Also, SSRIs are moderately to highly lipophilic (Kreke and Dietrich, 2008). Log *n*-octanol-water partition coefficients (K_{ow}) are a measure of the hydrophobicity or hydrophilicity of neutral compounds. The log K_{ow} of FLX, a neutral-weakly basic drug (Yargeau *et al.*, 2007), range from 3.82 to 4.67, showing that FLX is moderately lipophilic in the tissues of organisms

(Oakes *et al.*, 2010). Kreke and Dietrich (2008) have also reported FLX to accumulate extensively in fish tissue in comparison to other SSRIs. In fish, large volumes of FLX were measured in brain, liver and muscle tissues (Mennigen *et al.*, 2010), although distribution of FLX to other tissues was not tested. In addition, knowledge on the accumulation of FLX in fish tissue is important because it is most acutely toxic to non-target organisms compared to other human pharmaceuticals (Fent *et al.*, 2006).

Recently, Minagh *et al.* (2009) and Nakamura *et al.* (2008) confirmed that FLX and its metabolite can bioaccumulate in fish. This knowledge is vital as SSRIs, including FLX, are known to induce biological effects in fish, including delayed physiological and reproductive development (Foran *et al.*, 2004), reduced aggressiveness (Perrault *et al.*, 2003), and decreased feeding responses (Stanley *et al.*, 2007). A concentration of 3.2 µg/L FLX impaired egg production of zebrafish (*Danio rerio*) (Lister *et al.*, 2009) and as low as 0.1 µg/L FLX has altered plasma estradiol (E₂) concentrations in female Japanese medaka (*Oryzias latipes*) (Foran *et al.*, 2004). Research from Stanley *et al.* (2007) noticed a suppression of appetite from reduced feeding rates when fathead minnows were exposed to FLX. Alterations in the expression of neuropeptides involved in feeding as a result of FLX exposure were displayed as changes in food intake and weight gain in goldfish (*Carassius auratus*) (Mennigen *et al.*, 2010). In addition, FLX reduced the swimming and feeding behaviour of hybrid striped bass (*Morone saxatilis* × *M. chrysops*) (Gaworecki and Klaine, 2008) and it delayed the sexual development in maturing western mosquitofish (*Gambusia affinis*) (Henry and Black, 2008). However, these studies typically observed significant effects of FLX at concentrations far greater than those measured in the environment.

Research has indicated that FLX is toxic to parent organisms (Weston *et al.*, 2001; Kolpin *et al.*, 2002; Fong, 2001; Brooks *et al.*, 2003a; Richards *et al.*, 2003). However, there is limited research on the effects of FLX on offspring. Previous testing by Foran *et al.* (2004) noted several developmental abnormalities in Japanese medaka embryos from parents exposed to FLX treatments. My study will explore the embryotoxicity of FLX if it is maternally-transferred to oocytes via lipids during sexual maturation in causing developmental abnormalities noted by Foran *et al.* (2004).

Mechanism of Toxicity

In humans and vertebrates, SSRIs can act as substrates for specific cytochrome P-450 (CYP) isoenzymes in the liver. These substrates also inhibit activity, either by regulating their own metabolism via a negative feedback mechanism or by blocking the activity of other CYP isoenzymes (Kreke and Dietrich, 2008). Previous research has mainly focused on mammalian models, whereas the toxicity of SSRIs in teleost fish has not been widely studied. Currently, research on fish has detected the presence of CYP isoenzymes from the CYP3A family. These enzymes are primarily responsible for the metabolism of SSRIs and other substances (Kliwer *et al.*, 1998; Tseng *et al.*, 2005; Kashiwada *et al.*, 2007). The CYP3A enzymes, which belong to the cytochrome P-450 superfamily, are highly similar to enzymes in human and mammalian models as they display similar substrate specificity (Kreke and Dietrich, 2008; Celander *et al.*, 1996). By extension, SSRIs should also inhibit CYP isoenzymes in teleost fish. This could prevent SSRIs from being metabolized and subsequently affect steroid metabolism and hormonal homeostasis in fish (Kreke and Dietrich, 2008). In particular, FLX deserves special attention since inhibitory

effects on CYP-activity can persist for several weeks after exposure to FLX due to its long half-life in tissue.

Inhibition of CYP-activity from FLX exposure may decrease its rate of metabolism, which in turn would cause an increase in extracellular serotonin concentrations as it inhibits serotonin re-uptake at the presynaptic nerve (Schloss and Williams, 1998). Serotonin is one of the most ubiquitous neuromodulators in vertebrates. It is synthesized in the cells lining the gut, in the neurons of the hypothalamus which regulates the activity of the pituitary, and in the brainstem of all vertebrates (Nestler, 2001). After the formation of serotonin, the presynaptic neuronal membrane releases it into the synaptic cleft, where serotonin acts as a neurotransmitter. The cerebrospinal fluid that comes in contact with neurons in the hypothalamus will then distribute serotonin throughout the circulatory system. The presence of serotonin in the circulatory system allows it to act on several target tissues, including the pituitary (McEwen, 2002; Azmitia, 1999).

In fish, serotonin stimulates the secretion of gonadotropin releasing hormone (GnRH) in the pituitary, which in turn increases the release of gonadotrophin (Khan and Thomas, 1994). This is important because gonadotropin, a protein hormone, affect the normal growth and development of the teleost ovary (Goetz *et al.*, 1991; Lister and Van Der Kraak, 2008). An elevated concentration of gonadotropin increases steroidogenesis in the gonads, which successively increases circulating steroid hormone concentrations. (Somoza and Peter, 1991; Khan and Thomas, 1992; Senthilkumara *et al.*, 2001). The elevated concentrations of steroid hormones will increase the synthesis of prostaglandins (PG) which are needed for the maturation and ovulation of ovarian follicles (Goetz *et al.*, 1991; Lister and Van Der Kraak, 2008). In Japanese medaka (*Oryzias latipes*), serotonin

induced oocyte maturation in their germ cells (Iwamatsu *et al.*, 1993). Thus, it can be expected that FLX may affect reproduction and alter gametogenesis in fish organism.

FLX is moderately lipophilic and more FLX is known to accumulate in the tissue of exposed organisms compared to other antidepressants (Kreke and Dietrich 2008). Daniel and Wojcikowski (1997) also found that FLX can be trapped in cellular lysosomes, which may play a role in the retention of this drug in tissues. Thus, these studies show that FLX is relatively persistent in biological tissues and may bioaccumulate in the storage lipid of organisms.

Rationale and Study Objective

FLX is toxic to parent organisms, but there is limited research on how this may affect offspring. Foran *et al.* (2004) observed the development of Japanese medaka embryos, whose parents were exposed to FLX treatments. Several abnormalities were noted including: edema, curved spine, incomplete development (no pectoral fins, reduced eyes), and non-responsiveness to disturbances. These developmental abnormalities occurred 4 to 5 times more frequently in FLX treatments compared to controls. However, the model of toxicity in which FLX generated development abnormalities in embryos was not established.

My objective was to assess how FLX may adversely affect the offspring of exposed adult medaka. Two models of toxicity were proposed: (1) FLX causes mutation in offspring by acting directly on the germ cells of adults or (2) FLX is embryotoxic because it is transferred maternally to oocytes (via lipids) from the parent organism to embryos during oogenesis. Specifically, this study mimicked a one-time maternal transfer of compounds that was originally taken up by the parent organism. If FLX is maternally-transferred to

oocytes during sexual reproduction, I expected that signs of pathology will be observed in embryos exposed to FLX and predicted that exposure to graded doses will generate a dose-response relationship.

Japanese medaka was chosen as a model species as it is widely used to test contaminant-induced developmental effects (Metcalf *et al.*, 1999) and impaired reproductive capabilities (Arcand-Hoy *et al.*, 1998). It is easy to acquire and maintain in the lab, has a short incubation time of 12 days, reproduces all year round in a lab setting, and has a transparent chorion (egg envelope layer) which allows non-invasive observation during development.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

As there has been no previous research in modelling the maternal transfer of FLX, the majority of my project was devoted to methods development. Three possible exposure methods were considered: (1) waterborne exposure, (2) nano-injection, and (3) topical application.

Table 1. Considerations on Methods

	<u>Pros</u>	<u>Cons</u>
1. Waterborne Exposure	<ul style="list-style-type: none"> • Many eggs are exposed at once so less time-consuming process 	<ul style="list-style-type: none"> • Dose delivered into organism must be estimated • Expensive since more chemical is required • Continuous uptake of chemicals from egg stage to hatch (assumes FLX exposure is directly and entirely from the environment)
2. Nano-injection	<ul style="list-style-type: none"> • Known dose is delivered • Minimal chemical used and wasted • Affordable as less chemical is required • Simulates maternal transfer of contaminants (one-time dosage) 	<ul style="list-style-type: none"> • Individual exposure of eggs is a very time-consuming process • Estimating dose is complicated because injected solution is miscible with egg contents and cannot be seen
3. Topical Application	<ul style="list-style-type: none"> • Known dose is delivered • Minimal chemical used and wasted • Affordable as less chemical is required • Simulates maternal transfer of contaminants (one-time dosage) 	<ul style="list-style-type: none"> • Individual exposure of eggs is a very time-consuming process • Unknown quantity of chemicals uptaken across the chorion of the egg

Waterborne exposure is expensive because it requires static-renewal of solutions and large quantities of fluoxetine (Table 1). The renewal of test solution ensures continuous uptake of chemicals from fertilization to hatch. Consequently, this method does not represent a one-time maternal transfer of contaminants. On the other hand, nano-injections and topical application can model maternal transfer via exposing the egg with a one-time dosage; thus, I focused on developing these two methods for my project.

MEDAKA EGG PRODUCTION

Adult medaka (Golden strain from Carolina Biological Supply Company (Burlington, NC)) were reared in dechlorinated water at $26 \pm 1^\circ\text{C}$ in a re-circulated system with a photoperiod of 16 h light: 8 h dark. Fish were fed live brine shrimp (*Artemia salina*) (Ocean Star International, Inc.) three times a day and Nutrafin Basix staple flake food™ one hour after the onset of the photoperiod to induce oogenesis. Eggs were collected as soon as possible after fertilization by gently stripping them from females corralled in a dip net submerged in water. Medaka eggs were pooled from all the females in 15 aquaria containing four separate populations of the same strain of medaka (Golden strain). These populations consisted of fish from Arofish Supply Co., Hampton, NH; Trent University, Peterborough, ON; and Queen's University Kingston, ON. The collected eggs were placed in embryo rearing solution (ERS) (Appendix 3), a combination of salts and methylene blue mold inhibitor (Carolina Biological Supply Co., Burlington, NC), and examined microscopically to determine if they were viable.

STANDARD BIOASSAY PROCEDURES

The typical bioassay procedure is described below; however, certain aspects of these procedures were modified or used based on the particular needs of the method. These

standard procedures were used for both nano-injection (method 2) and topical application (method 3) unless otherwise noted.

1. Needle Preparation

Quartz glass needles with filaments (QF 100-70-10, Sutter Instrument Co., Novato, CA) were prepared at the United States Geological Services laboratory in Columbia, MO. At Queen's University, the needles were beveled at a 20° angle on a nanopipette beveller (BV-10 Sutter Instrument Co., Novato, CA). Beveled needles were coated with Sigmacote® (a silicon solution in heptane, Sigma-Aldrich St. Louis, MO), to reduce clogging by egg yolk. The quartz needles were back-loaded with 8 µL of dosing solutions as close to the injection time as possible using an Eppendorf microloader (detailed in Appendix 4).

2. Needle Calibration

Nano-Injection

Each injection needle was calibrated by dispensing a small volume of the exposure solution into ultra-pure water or corn oil to measure the diameter of the spherical droplet using the microscope's eyepiece micrometer. The target injection volume is typically 0.1% of the egg's internal volume to avoid causing physical stress to the egg (Walker *et al.*, 1996). The injection volume was estimated by assuming that a medaka egg is spherical ($\pi d^3/6$) in shape. A medaka egg was approximately 1.25 mm in diameter, and the egg volume assumed to be 1023 nL (Iwamatsu, 2004). An injection of 0.1 % of the egg volume would equal to a dosing volume of 1.0 nL. This dosing volume can be injected through the chorion (egg envelope layer) of the embryo into the yolk matrix of the egg. By adjusting the injection duration and pressure, the dosing volume could be standardized every time. During this process, the balance pressure of the Pico-Injector was maintained greater than

the internal pressure of the egg to prevent vitelline fluid from entering the injection needle upon insertion into the egg.

Topical Application

A small volume of the dosing solution was dispensed from the quartz needle so that a droplet of the dosing solution could be seen hanging from the tip of the needle. Calibration of the droplet was achieved by estimating the diameter of the spherical droplet using the microscope's eyepiece micrometer. The exposure volume was estimated by assuming that a medaka egg is spherical ($\pi d^3/6$) in shape. Assuming the egg volume was 1023 nL, a 10.0 nL dosing volume is 1 % of the egg volume. The 10.0 nL dosing volume was applied by placing the tip of the needle containing the solution onto the top of the medaka egg. By adjusting the duration and pressure of the Pico-Injector, the dosing volume could be standardized each time. The balance pressure of the Pico-Injector was kept very low to prevent the solution from dripping from the tip of the needle.

3. Egg Preparation and Exposure

Nano-Injection

Eggs were injected according to Åkerman & Balk (1995) and Walker *et al.* (1996). Newly fertilized eggs were immobilized in holes punched in a layer of agar. Eggs were covered with ERS for 10 to 20 minutes. Viable eggs (did not stain blue) were injected individually 2 to 4 hours after fertilization using the needle mounted in a micromanipulator (MX110R, Siskiyou Corporation, Grants Pass, OR) at a 60° angle under a stereomicroscope and a Pico-Injector (PLI-100, Harvard apparatus St. Laurent, QC). The dosing solution was injected through the chorion of the embryo into natural oil droplets at the vegetal pole (the

portion of an egg that contains most of the yolk) to ensure that the solution would be incorporated into the developing embryo.

Topical Application

Square petri dishes were lined at the outer rim with wet paper towels immersed in ultra-pure water to ensure that the medaka eggs were kept moist throughout the exposure period. Newly fertilized eggs (2 to 4 hours after fertilization) were placed into individual square grids on the surface of a square petri dish using a transfer pipette. Residual water around the medaka eggs was removed using a transfer pipette to enable the topical application. A micromanipulator at a 60° angle was used under a stereomicroscope, along with a Pico-Injector, to apply the dosing solution onto the top of the medaka egg. Medaka eggs were allowed to sit on the square petri dish for 30 minutes to 1 hour.

4. Egg Rearing

The exposed eggs were transferred to 20 mL borosilicate glass scintillation vials (Fisher scientific) with 15 mL of clean ERS (dissolved oxygen content of 8.7 mg/L, conductivity of 180 $\mu\text{s}/\text{cm}$ and pH of 7.76). The vials were capped with Teflon-lined lids and placed on a Mistral Multi-mixer (Lab-line instruments, Inc.) set to roughly 90 revolutions per minute (rpm) and incubated at a room temperature of $26 \pm 1^\circ\text{C}$ with a photoperiod of 16 h light: 8 h dark and 24 h renewal of ERS. The duration of the bioassay was 12 days post fertilization (dpf), to ensure that most embryos had hatched by this time; day 0 is considered the day the eggs were fertilized and exposed. Every day after exposure, the embryos were observed for mortalities, signs of pathology, and hatchlings. On the twelfth day of the bioassay, the embryos were scored for signs and severity of embryotoxicity and sacrificed by an overdose of an anesthetic, tricaine methanesulfonate (MS-222, Sigma-Aldrich).

5. Scoring Eggs For Embryotoxicity

On day 12 of each experiment, eggs were inspected under a Leica MZ95 (Meyer Instruments, Houston TX) compound microscope and images were taken using SPOT advanced imaging software (Ver. 4.7, Diagnostic instruments Inc. Sterling Heights, MI). Double blind scoring was performed to reduce bias by covering the treatment vials with tin foil and randomly assigning a letter of A to J to each vial. The vials were shuffled and selected randomly for scoring (not scored in order from A to J). Medaka eggs were scored for signs and severity of embryotoxicity, as modified from Kennedy (2010). Signs of pathology included: pericardial and yolk sac edema, abnormal eye development, fin rot, spinal deformities, body and yolk haemorrhaging, and craniofacial deformities. These signs were given a score between 0-3 (3 being the worst case) or 0-1 (presence or absence). Yolk sac (YSE) and pericardial edema (PE) were scored on a 0-3 scale because the embryos showed a broad range of responses allowing a discrimination of mild and severe responses. Abnormal eye development (AED), fin rot (FR), spinal malformations (SM), body hemorrhaging (BH), and craniofacial malformations (CF) was scored on a scale of 0-1 (presence or absence) due to the difficulty in judging a gradation in their severity. The maximum possible score for each fish was 11, the sum for all signs of pathology. Assymetry (ASYM) and embryo body opacity (EBO) were also observed in this study but were not included in the embryotoxicity scoring because they were observed after the completion of the scoring template.

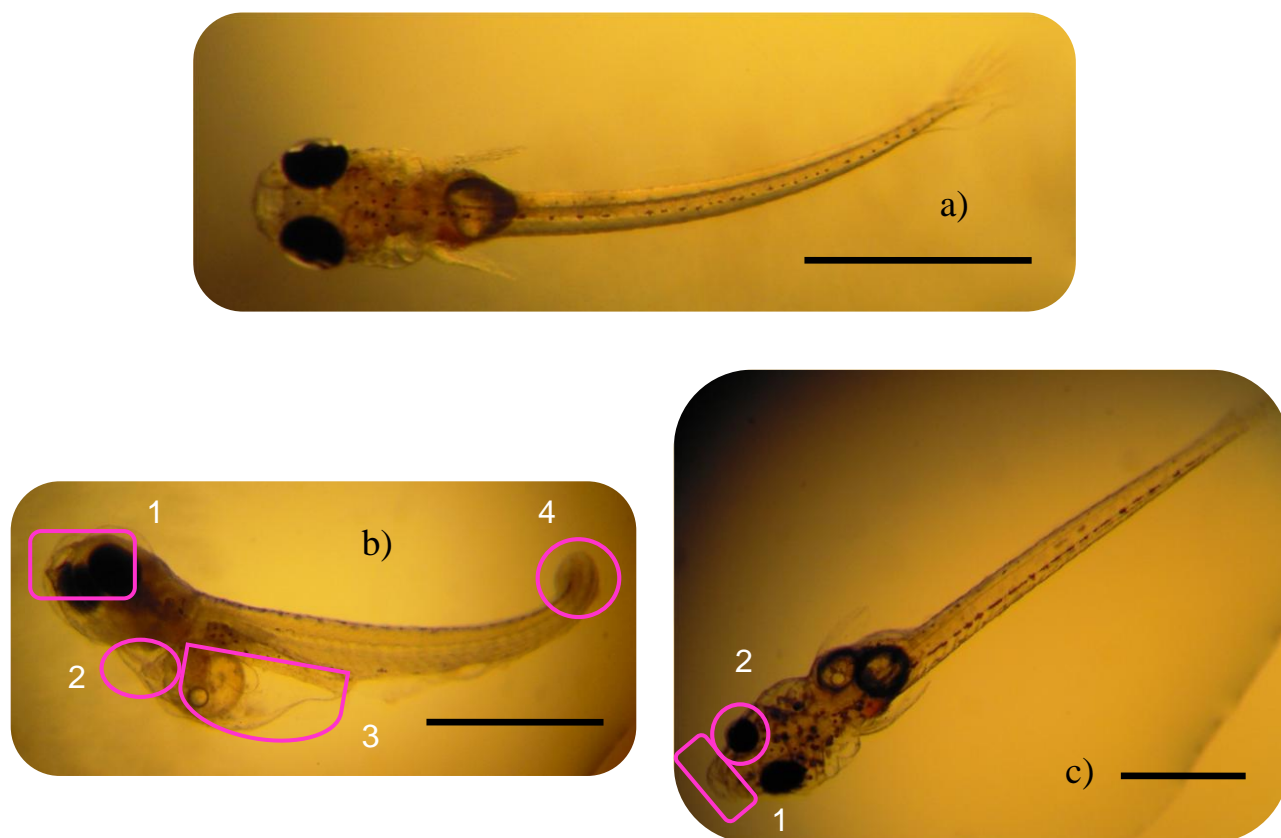


Figure 1: Images of medaka embryos taken 12 dpf and after topical application of FLX.

Scale bars represent 1 mm. (a) Normal embryo, straight spine, normal eye development

(equal eye size) and absorbed yolk sac. (b) Embryo exposed to 0.01 µg/mg FLX exhibiting

1) craniofacial malformations (jaw malformation and blunt nose), 2) pericardial edema, 3)

yolk sac edema, 4) spinal deformities and fin rot. (c) Embryo exposed to 0.001 µg/mg FLX

exhibiting 1) craniofacial malformations (elongated jaw) and 2) abnormal eye development

(unequal eye size). Asymmetry of the body can also be seen though it was not scored for as a sign of toxicity.

6. Calculating Embryotoxicity Score

Individual fish scores for signs of pathology were averaged to give embryotoxicity scores for live fish in each treatment. Mortalities after day 0 were assumed to be associated with embryotoxicity due to FLX exposure. Dead fish were assigned the maximum score for embryotoxicity (11) plus 0.5; therefore the highest possible embryotoxicity score when mortality was considered was 11.5.

7. Calculating Prevalence for Signs of Pathology

The prevalence is the percentage of the sum of the presence for each sign of pathology for all fish in each treatment normalized to the total number of fish in the FLX treatment. Mortalities were not included in this calculation as they were not recorded for signs of pathology. This creates a measure of the prevalence on a 0 to 100% scale in which the highest possible prevalence for each sign of pathology was 100%.

METHOD 2: NANO-INJECTION

This method has many advantages (Table 1) mainly because it simulated the maternal transfer of contaminants with a one-time dosage. Past nano-injection experiments from Kennedy (2010) used triolein (oil) to deliver hydrophobic compounds. Oil facilitates the estimation of injection volume and dose because oil droplets can be easily observed in the egg (Figure 1) and the injection volume can be measured using the formula for the volume of a sphere. Information from Sigma-Aldrich stated FLX to be soluble in water at 4 mg/mL and DMSO at ≥ 5 mg/mL, while it is insoluble in hexane, benzene and other non-polar solvents. This caused difficulties in calibrating the needle and verifying a consistent injection volume because polar or aqueous solutions (water and DMSO) will disperse instead of forming a spherical droplet similar to natural oil droplets within the egg (Figure

2). Thus, various aqueous solutions were tested to quantify the injection volume and resolve this issue.

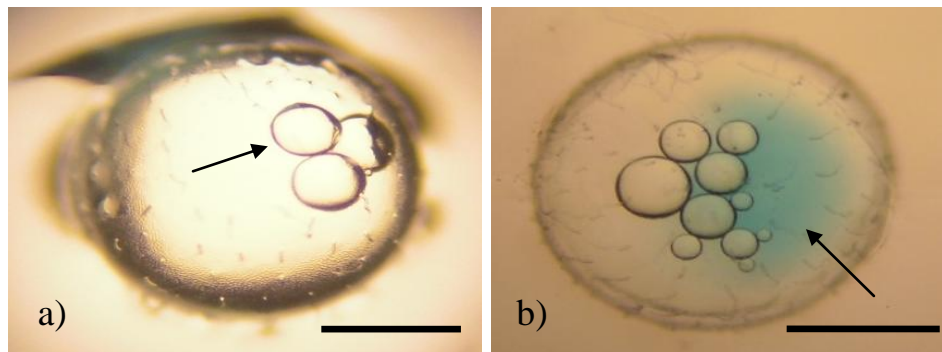


Figure 2. (a) Nano-injection of a spherical triolein droplet (noted by the black arrow) in a medaka egg. (b) Nano-injection of an aqueous solution containing methylene blue (noted by the black arrow). The solution dispersed in the medaka egg, as seen by the dark area to the right of the natural lipid droplets, instead of forming a sphere. Scale bars represent 0.5 mm.

Aqueous Solutions Tested

1. 0.9% Saline solution with methylene blue (Figure 2)

The sodium chloride and methylene blue (Carolina Biological Supply Co., Burlington, NC) solution was successfully calibrated in corn oil, but it dispersed in the medaka egg and did not form a spherical droplet once injected. Thus, the volume injected could not be quantified.

2. Carboxymethylcellulose (0.125, 0.25, 0.5, 1, 2, 3, 4, 5, and 10%) with methylene blue

Carboxymethylcellulose was obtained from Sigma-Aldrich (St. Louis, MO). This non-toxic compound has a density of 1.59 g/cm^3 and increased the viscosity of the solution. Methylene blue was mixed with carboxymethylcellulose to aid in quantifying the injection

volume. Solutions of 0.125 to 2% carboxymethylcellulose in ultra-pure water could be loaded into the injection needle, but not solutions of 3% to 10% carboxymethylcellulose, which coagulated and could not be loaded into the needle. Droplet sizes using 0.125 to 2% carboxymethylcellulose solution were successfully calibrated in corn oil, but once injected into the medaka egg, the solutions dispersed and injection volumes could not be quantified.

3. Polyethylene glycol (PEG) - 200, 400 and 600 with methylene blue

PEG with molecular weights of 200, 400 and 600 were obtained from Sigma-Aldrich (St. Louise, MO), at 20°C each had densities of 1.1238, 1.1255, and 1.1258 g/mL, respectively. PEG is a non-toxic polymer known to be soluble in aqueous and organic solvents. However, it could not be calibrated in ultra-pure water or corn oil, and it dispersed when injected into medaka eggs.

4. Castor oil, olive oil, and mixtures of both with fast green dye

Castor oil (a polar solvent) and olive oil (a non-polar solvent) were obtained from Shoppers Drug Mart (Kingston, Ontario). Fast green dye (dye content $\geq 85\%$) from Sigma-Aldrich (St. Louis, MO) was added to the solution as a visual indicator of droplet volume. Takeuchi (1965) found that a mixture of olive oil and castor oil is relatively non-toxic and that this mixture was a good solvent for lipids. The olive oil with fast green dye could be calibrated in ultra-pure water, but it dispersed in the medaka egg once injected. Castor oil with fast green dye could not be calibrated in ultra-pure water or corn oil, and it too dispersed in the medaka egg once injected. The mixtures of 1:1, 1:2, and 1:3 (castor oil: olive oil) with fast green dye did not mix uniformly, and were not suitable for calibrating and quantifying injection volume.

Conclusion

In conclusion, all the solutions tested were unsuccessful for calibrating and quantifying the injection volume in the medaka egg; thus, nano-injection was not ideal for modelling maternal transfer of FLX.

METHOD 3: TOPICAL APPLICATION

As the nano-injection method was unsuccessful, the third method of topical application was attempted to model maternal transfer of contaminants. It has many advantages, including easy calibration of the injection volume.

Before FLX doses were tested by topical application, a quality control test was done to ensure that all chemicals topically applied with a FLX and DMSO solution would be incorporated and absorbed by the medaka egg. DMSO was selected because it is a solvent carrier which increases membrane permeability and facilitates the absorption of chemicals (Helmstetter and Alden III, 1995b). A solution containing DMSO mixed with methylene blue was applied to the surface of the egg, and after 30 minutes, all of the solution was absorbed as indicated by a blue tinge throughout the egg. Thus, it was assumed that DMSO could carry the FLX with 100% efficiency from the surface of the medaka egg, past the chorion, and into the yolk sac of the medaka egg with no loss of this drug during the topical application process.

Chemical Preparation

A stock solution of fluoxetine hydrochloride (≥ 98 % pure), was purchased from Sigma-Aldrich (St. Louis, MO). A dosing solution was prepared by dissolving FLX in dimethylsulfoxide (DMSO) followed by serial dilution to get eight doses (10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0.000001 $\mu\text{g}/\text{mg}$). Also, 100% DMSO and ERS solutions were

prepared to act as controls. DMSO ($\geq 99.9\%$ pure) was purchased from Sigma- Aldrich (St. Louis, MO). The doses were selected based on a practical dilution of a limited quantity of chemical on the assumption that standard aquatic toxicity tests suggested adverse effects of FLX in fish at a waterborne exposure as low as $1\ \mu\text{g/L}$ (Schultz *et al.*, 2010). A bioconcentration factor (BCF) of 74 for FLX was used to estimate the dose of FLX that will cause adverse effects in medaka eggs to generate the testing dose range (detailed calculations in Appendix 5) (Paterson and Metcalfe, 2008). All solutions were stored in 5 mL amber vials at 4°C . Before use, the vials were removed from the fridge and left at room temperature overnight.

Experimental Setup

Medaka eggs ($n=10$ for each treatment) were allowed to sit on the square petri dish for 30 minutes or 1 hour to test whether the severity of pathology was affected by exposure duration. There were two replicates for each exposure period for a total (N) of 20 medaka eggs per treatment.

Statistics

The percent normal and percent mortality graphs, prevalence graphs, linear regressions, and ANOVA analysis were constructed with SigmaPlot 11.0. Analysis for the significance of the slopes was performed with GraphPad Prism 5.

RESULTS

One-Hour Fluoxetine Exposure

The proportion of fish that appeared normal (% normal) for ERS and 100% DMSO were both 79%. An evaluation of the % normal across various treatments yielded a significant decrease in normal Japanese medaka as FLX dose increased ($p = 0.005$). A strong negative linear regression [$y = -6.567 (\log x) + 16.290$] was fitted through all the data points to obtain a coefficient of determination (r^2) of 0.76.

The percent mortalities for the controls, ERS and DMSO, were 21% for both. There was no statistically significant ($p > 0.05$) relationship between percent mortality and doses of FLX ($r = 0.044$ for a linear regression).

A one-hour exposure to FLX caused signs of abnormalities in over 50% of the Japanese medaka for each FLX concentration. This suggests that the abnormality may have been caused by severe stress from the duration of medaka eggs out of water; thus, these results did not provide useful information on the toxicity of fluoxetine exposure.

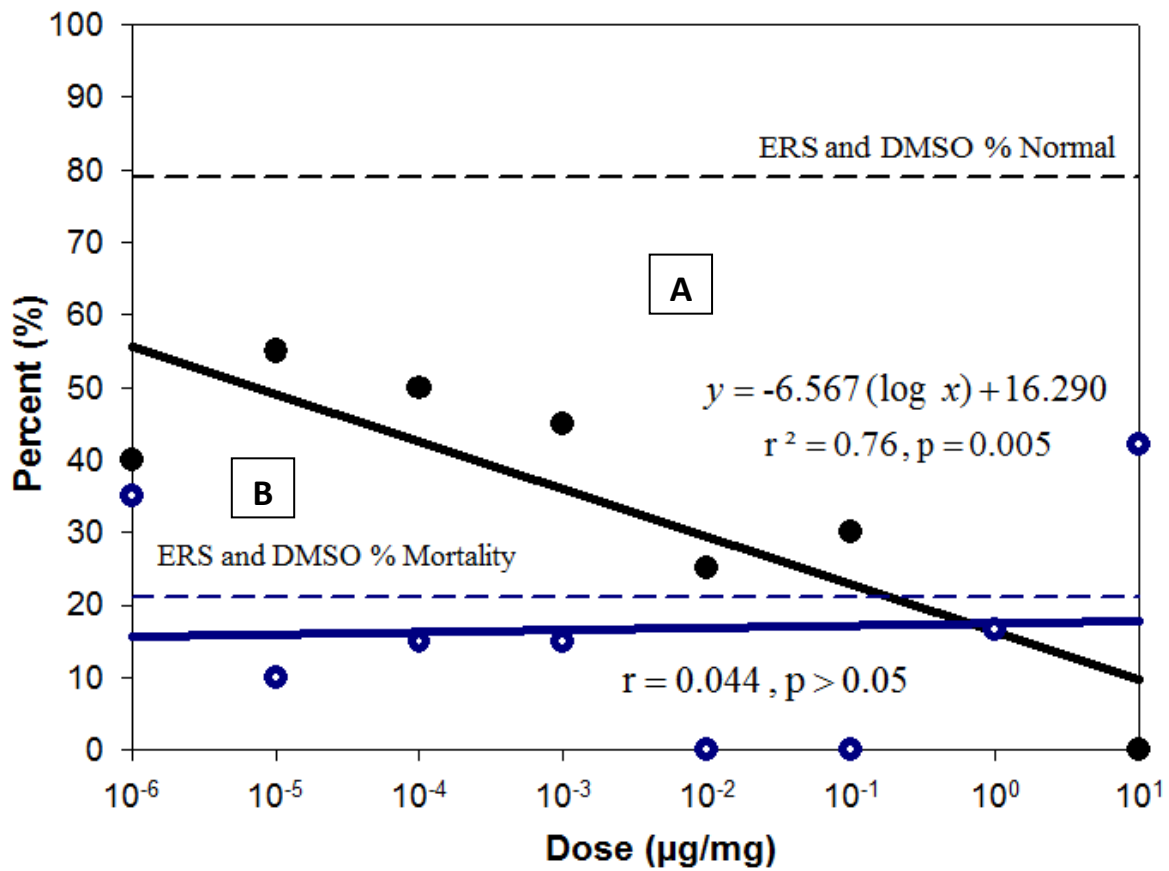


Figure 3. The effect on medaka embryos of a one-hour exposure to graded dose of fluoxetine. Dashed lines represent the response of controls. (A) Percent normal for doses of fluoxetine. A linear regression ($r^2 = 0.76, p = 0.005$) was applied to all filled black circle data points. (B) There was no linear relationship ($r = 0.044, p > 0.05$) between percent mortality and dose of fluoxetine (open blue circles).

Thirty-Minute Fluoxetine Exposure

The percent normal for ERS and 100% DMSO were 95% and 80%, respectively.

An evaluation of the % normal yielded a strong negative linear relationship [$y = -6.905 (\log x) + 37.738$, $r^2 = 0.61$, $p = 0.022$] between the percentage of normal Japanese medaka and the dose of FLX.

The percent mortality for both the ERS and 100% DMSO controls was 5%. The average rate of mortality was 2.5% for FLX dose between 1.0×10^{-6} to 1.0×10^{-1} $\mu\text{g}/\text{mg}$. The highest percent mortality (20%) was observed at the two highest doses, 1 and 10 $\mu\text{g}/\text{mg}$. No statistical trend between percent mortality and dose of FLX was observed, but an increase in percent mortality for the two highest doses suggests a threshold was passed. A dotted straight line was used to represent the elevated percent mortality after passing the threshold.

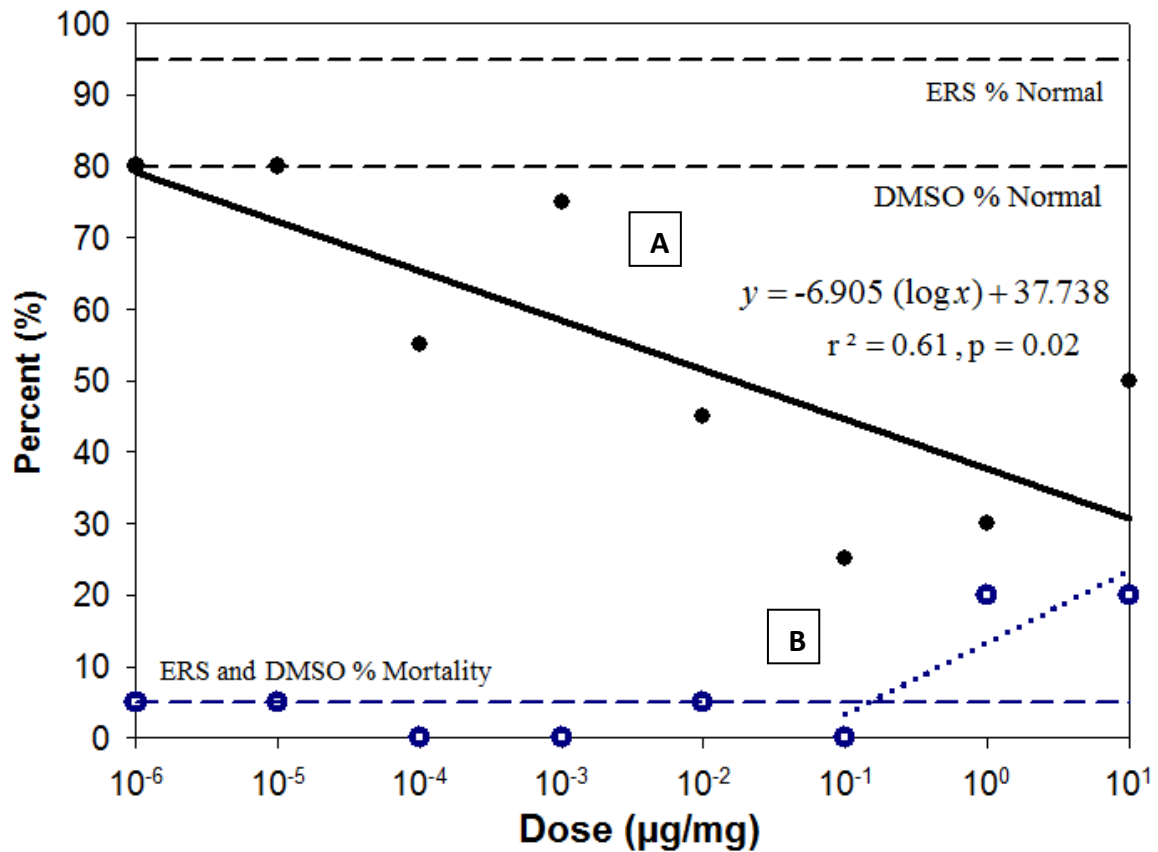


Figure 4. The effect on medaka embryos of a thirty-minute exposure to graded dose of fluoxetine. Dashed lines represent the response of controls. (A) Percent normal for doses of fluoxetine. A linear regression ($r^2 = 0.61$, $p = 0.02$) was applied to all filled black circle data points. (B) Percent mortality for doses of fluoxetine. A dotted straight line was applied to FLX doses between 0.1 to 10 $\mu\text{g}/\text{mg}$ to indicate a possible increase in percent mortality after a threshold was passed (open blue circles).

Prevalence of Signs of Pathology and Other Observations for 30 Minute Fluoxetine

Exposure

For most signs of pathology, the prevalence for controls was 0 for both ERS and DMSO, with the exception that the DMSO control for body hemorrhaging was 0.16 and the ERS control for embryo body opacity was 0.05. An evaluation of yolk sac edema (YSE), pericardial edema (PE), abnormal eye development (AED), fin rot (FR), spinal malformations (SM), and craniofacial malformations (CF) across various treatments showed basal levels of prevalence for all signs of pathology. There was no significant linear relationship between the dose of FLX and the prevalence of signs of pathology ($p > 0.05$). The range of coefficients of determination for all these signs of pathology was 0.02 to 0.11. However, a moderate positive linear relationship ($r = 0.67$) was observed for body hemorrhaging (BH), though the prevalence of BH and doses of FLX did not differ statistically ($p = 0.067$). In addition, an inverse relationship between BH and % normal after a 30 minute exposure was observed. This is important since data points for % normal after a 30 minute exposure (Figure 4) incorporated the results for all other signs of pathology. Thus, it appears that BH is the sign of pathology that most contributed to the decrease in percentage of normal Japanese medaka (Figure 4).

Asymmetry (ASYM) and embryo body opacity (EBO) were also observed throughout all FLX treatments. ASYM was recorded when there was an unequal proportion between the left-right sides of the embryo when observed from a frontal plane. EBO was noted when a lack of transparency was observed throughout the tissue of the embryo. However, these two signs of pathology were not included in the embryotoxicity scoring because these signs were observed after the completion of the scoring template. A low

prevalence of ASYM was observed throughout all FLX treatments which showed there was a poor relationship ($r^2 = 0.1$) between the dose of FLX and the prevalence for ASYM. A significant exponential relationship ($r^2 = 0.94$, ANOVA modified simple exponent $p = 0.001$) was observed between the dose of FLX and the prevalence of EBO. Thus, the prevalence for EBO may be a sign of pathology caused by FLX exposure.

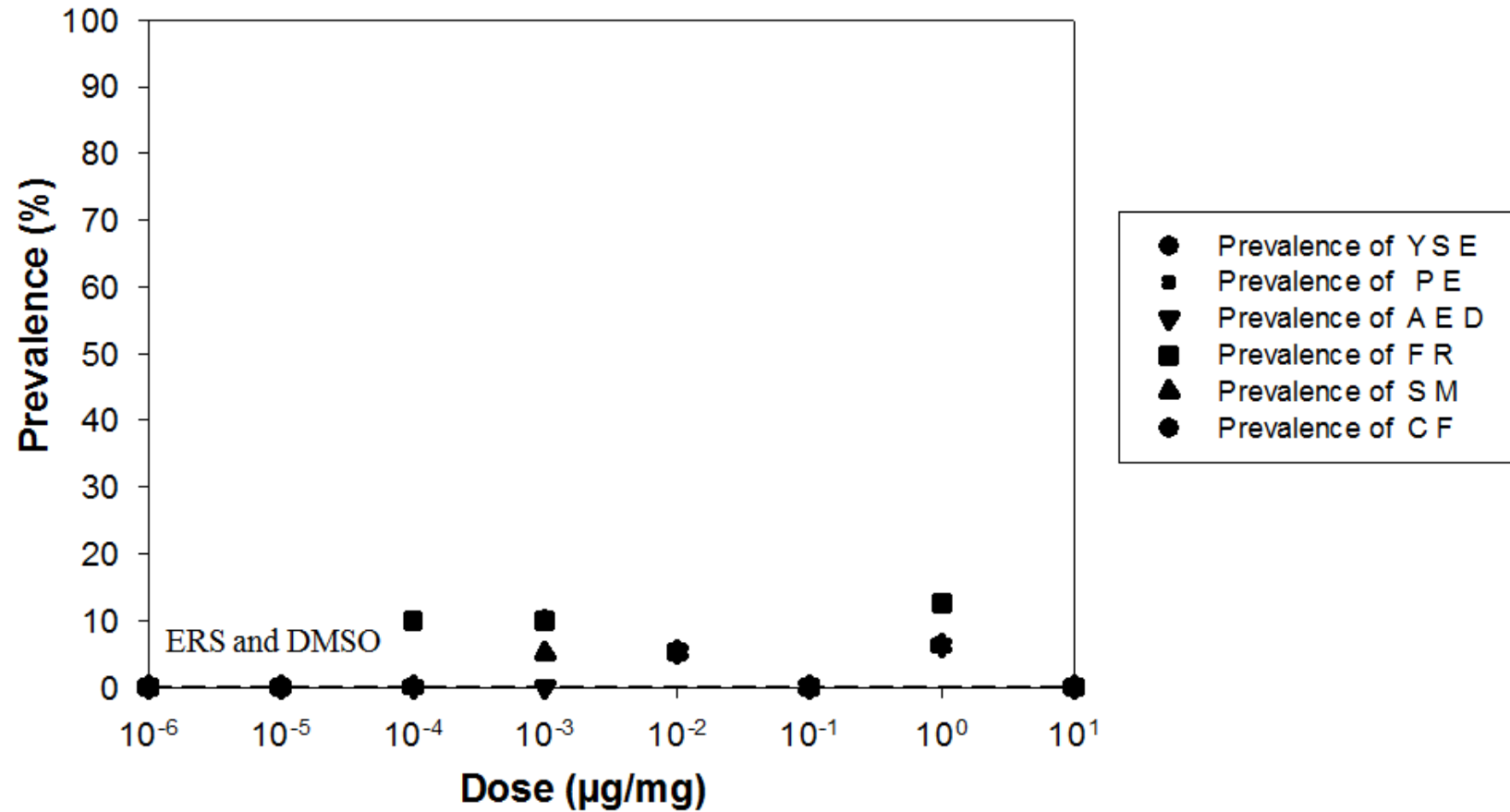


Figure 5. Prevalence of signs of pathology in Japanese medaka embryos after 30 minute exposure to fluoxetine and 12 d development in clean water. Dashed line represents the response of controls. No statistically significant correlation ($p > 0.05$) was found between doses of fluoxetine and prevalence of yolk sac edema (YSE), pericardial edema (PE), abnormal eye development (AED), fin rot (FR), spinal malformations (SM), and craniofacial malformations (CF).

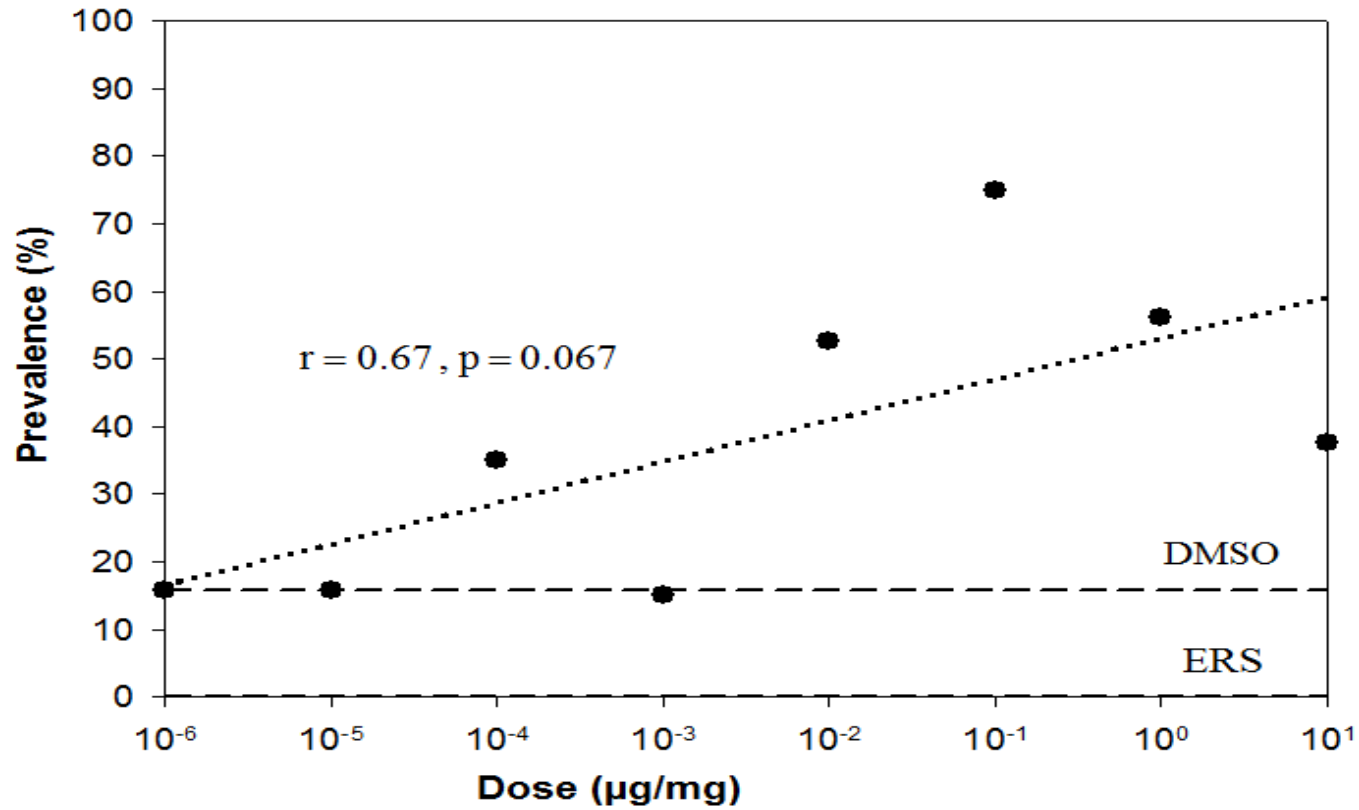


Figure 6. Prevalence of body hemorrhaging in Japanese medaka after a 30 minute exposure to fluoxetine and 12 d development in clean water. Dashed lines represent the response of controls. A dotted line was used to show a moderate linear relationship ($r = 0.67$) between doses of fluoxetine and prevalence for body hemorrhaging (BH) as the correlation was not statistically significant ($p = 0.067$).

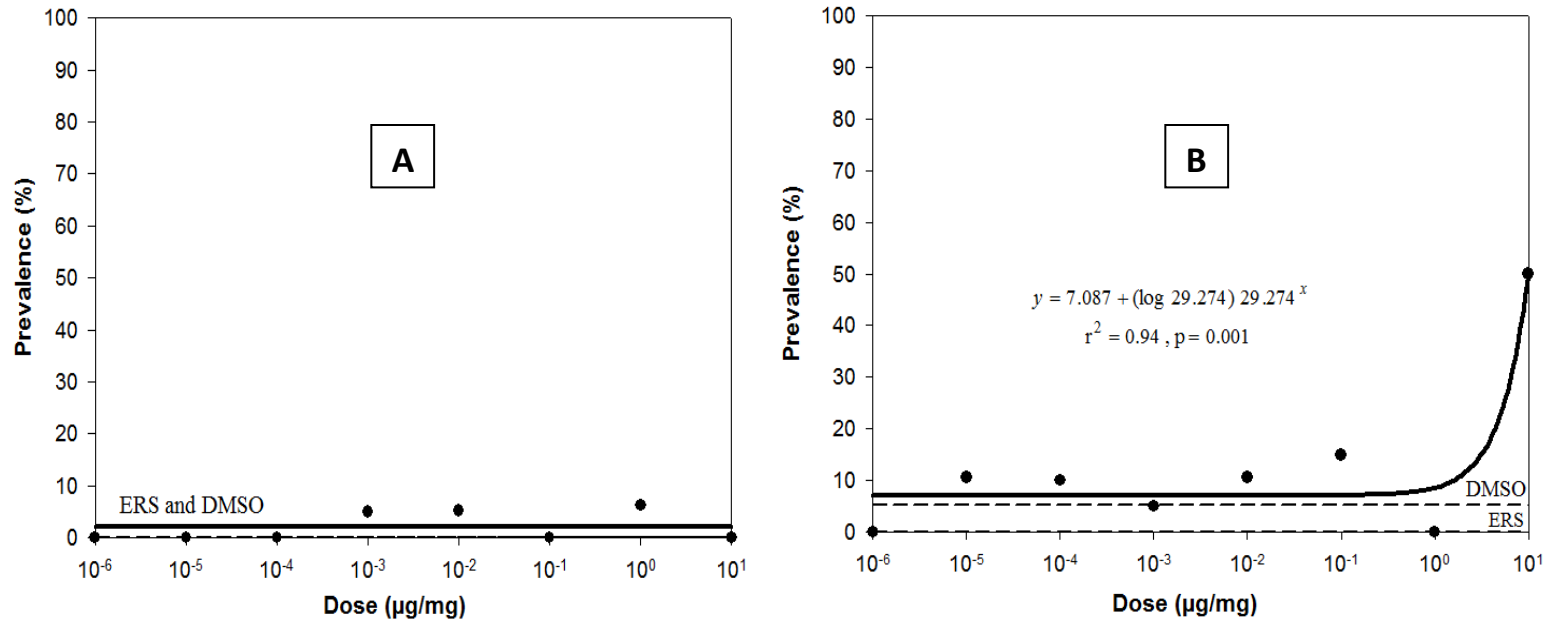


Figure 7. Prevalence of other signs of pathology in Japanese medaka embryos after a 30 minute exposure to fluoxetine and 12 d development in clean water. Dashed lines represent the response of controls. (A) Prevalence of asymmetry (ASYM). The solid line represents the average prevalence (2.06%) of ASYM for all doses of fluoxetine (FLX) as the results were not statistically significant ($p > 0.05$). (B) Prevalence of embryo body opacity (EBO). A significant exponential relationship ($p = 0.001$), represented by a solid line, was observed between doses of FLX and prevalence of EBO.

Table 2. Toxicity of Fluoxetine to Japanese medaka

<u>Response</u>	<u>Prevalence Range</u>	<u>ED50</u>
YSE	0 – 10.00 %	> 10 µg/mg
PE	0 – 10.00 %	> 10 µg/mg
AED	0 – 6.25 %	> 10 µg/mg
FR	0 – 12.50 %	> 10 µg/mg
SM	0 – 6.25 %	> 10 µg/mg
BH	0 – 75.00 %	3.0×10^{-3} µg/mg
CF	0 – 10.00 %	> 10 µg/mg
ASYM	0 – 6.25 %	> 10 µg/mg
EBO	0 – 50.00 %	≥ 10 µg/mg

The range of prevalence was determined from observing the minimum and maximum prevalence in all FLX treatments for each sign of pathology (Figure 5-7). The median effective dose that induces 50% of the maximal effect (ED50) for BH was approximated to be 3.0×10^{-3} µg/mg, while all other signs of pathology had ED50s > 10 µg/mg.

DISCUSSION

This study obtained several important findings for medaka embryos: (1) a 1 hour exposure induced severe stress as over 50% of the embryos had signs of abnormalities for all FLX treatments, and no correlation was observed between the dose of FLX and percent mortality, (2) percent normal significantly decreased as FLX doses increased for the 30 minute exposure, (3) basal levels of percent mortality were observed between 1.0×10^{-6} to 1.0×10^{-1} $\mu\text{g}/\text{mg}$, while the highest percent mortality (20%) was noted at 1 and 10 $\mu\text{g}/\text{mg}$ for the 30 minute exposure, (4) based on embryotoxicity scoring, the highest prevalence was observed for body hemorrhaging compared to other signs of pathology, (5) there was a moderate correlation between the prevalence for embryo body opacity and dose of FLX, though body opacity was not included in embryotoxicity scoring, and (6) the ED50 for body hemorrhaging was estimated to be 3.0×10^{-3} $\mu\text{g}/\text{mg}$, while all other signs of pathology had ED50s > 10 $\mu\text{g}/\text{mg}$. These findings have important implications for the embryotoxicity of FLX by modelling maternal transfer to oocytes during sexual maturation.

Maternal transfer of embryotoxic environmental contaminants is one model of toxicity proposed to contribute to the developmental abnormalities seen in the embryos of Japanese medaka. In this model, if FLX is accumulated in the fat stores of the female medaka, these lipids containing FLX are expected to transfer from the muscle to the developing reproductive organs (oocytes) during sexual maturation. This causes increased exposure of FLX to offspring as ovaries contain a high lipid content (Yamamoto and Yoshioka, 1964). This has important environmental implications as this will result in sub-lethal and lethal toxic responses in the embryos. The primary objective of this study was to determine whether FLX may be embryotoxic when maternally-transferred to embryos of

exposed adult medaka. A dose-response relationship was expected between percent normal and graded doses of FLX at the 30 minute exposure. From the results, a strong negative linear correlation ($p = 0.02$) was observed between dose and the percentage of normal embryos following a 30 minute exposure to externally applied FLX. An increase in prevalence for body hemorrhaging was the sign of toxicity that most contributed to the decrease in percent normal. Therefore, it can be expected that if FLX is maternally-transferred to oocytes via lipids during sexual maturation, it will cause embryotoxicity.

Limitations of Experimental Design

Topical application is a passive method used to deliver a significant dose across the chorionic membrane and into the developing embryo (Helmstetter and Alden III, 1995). Preliminary studies conducted by Maccubbin et al. (1987) and by Helmstetter and Alden III (1995a) proved this method to be a useful assay for single toxicants. As such, it was used to model a one-time maternal transfer of FLX to oocytes via lipids during sexual maturation. However, there are limitations with using this method, with three factors deserving special consideration.

1) *Assumptions*: Two assumptions were made in this study: (1) topical application was 100% efficient in delivering the dose of FLX to medaka eggs and (2) DMSO does not interact with FLX to cause toxic effects or to actively alter its lipid solubility characteristics.

Previous experimentation from Helmstetter and Alden III (1995a) determined a relationship between permeability factor (PF) and the physico-chemical coefficient for lipid affinity, using $\log K_{ow}$. This relationship was determined by employing a medaka embryo-larval assay (MELA) topical treatment technique. A regression line $PF = 11.1 \log K_{ow} + 3.97$ ($r^2 = 0.96$) suggested that the transport of toxicants was governed by their physical and

chemical properties (Helmstetter and Alden III, 1995a). By extension, FLX was estimated to have a PF of 50%, based on a measured log K_{ow} value of 4.05 (Adlard *et al.*, 1995). It can be interpreted that approximately 50% of FLX was transferred through the chorion and into the medaka egg. However, the MELA technique involved a one-minute exposure, while the exposure period for this study was 30 minutes. The longer exposure period used in this study may have increased the permeability factor of FLX to allow a higher transfer of the substance into the egg. A quality control experiment using a solution of DMSO with methylene blue and a 30 minute exposure was conducted to ensure that chemicals topically applied were incorporated and absorbed by the medaka egg. This was confirmed when all of the solution was absorbed, as indicated by a blue tinge throughout the egg. However, as the internalized dose in the egg was not quantified, only nominal doses were expressed in this experiment.

In addition, the MELA topical application technique used medaka eggs 48 hours post-fertilization. At this stage, the early heart has begun to form and contract, the tail bud has formed, the formation of optic lobes has begun and oil globules have coalesced into a single drop (Helmstetter and Alden III, 1995b). This may have affected the permeability and physico-chemical properties of the toxicant as later stages of fish embryos can biotransform and eliminate toxicants (Hendricks, 1981; Maccubbin and Black, 1986). In the present study, medaka eggs were exposed a few hours post-fertilization, when eggs are in the early cleavage stages of development. As this experiment models the maternal transfer of FLX to oocytes during sexual maturation, exposing eggs shortly after fertilization will more accurately simulate the effects of maternal transfer of FLX contaminants to offspring. Thus,

to ensure FLX is completely internalized in newly fertilized egg after a 30 minute exposure, further studies are needed to look at quantifying the dose after this exposure duration.

Secondly, DMSO was assumed to be a solvent carrier that delivered FLX into medaka eggs without causing structural alteration of the test substance and of the egg membrane, or acute or sublethal effects. DMSO has the ability to permeate biological membranes without causing significant damage to its structural integrity (Rammler and Zaffaroni, 1967). Past egg-exposure experiments that used DMSO as a solvent carrier found no toxicity to developing fish embryos (Helmstetter and Alden III, 1995b; Black *et al.*, 1988; Metcalfe *et al.*, 1988; Robertson *et al.*, 1988). These studies showed that DMSO is relatively non-toxic, though there is currently no toxicity information on the interactions of DMSO and toxicant mixtures. Therefore, future studies need to look at whether DMSO plays a role in changing physico-chemical properties of toxicants to produce signs of pathology in embryos.

2) *Quantity and Duration of FLX exposure*: The MELA technique involved a topical delivery of 100 nL of DMSO, while the present study delivered a dosing volume of 10 nL. Preliminary testing to determine the optimal dosing volume showed that 100 nL exceeded the surface area of the egg, i.e. the excessive volume would spill over the egg so that portions of each dose were not incorporated. In addition, the 100 nL DMSO dosing volume also caused a significant increase in embryo mortality. Conversely, a 10 nL DMSO dosage appeared to be completely absorbed in the egg and did not affect basal levels of mortality.

Low levels of mortality were observed after a 30 minute exposure to DMSO, while significant levels of mortality were observed after a 1 hour exposure to DMSO. This showed that longer exposure durations induced severe stress on medaka eggs. Therefore,

the selected quantity and exposure duration (30 minute) of DMSO was unlikely to contribute to embryotoxicity. However, further research is needed to determine the most optimal dosage and exposure period, and to quantify the dose that has been internalized by the medaka egg.

3) *Duration of Rearing Period*: Medaka embryos were reared for 12 days after which pathology was scored. Hatching time for medaka can vary between 8 to 35 days, with a median hatching time of 11 days (Teather *et al.*, 2000). Scoring medaka embryos on Day 12 allows most medaka embryos to be hatched. However, variability in the results may be seen as not all medaka are at the same stage of development when scored. To reduce this bias, future studies should isolate the hatched embryos and score embryotoxicity exactly two days after hatch.

Abnormal Signs of Pathology

Seven signs of pathology were found in medaka embryos exposed to FLX treatments: yolk sac edema, pericardial edema, abnormal eye development, fin rot, spinal malformations, body hemorrhaging, and craniofacial malformations (blunt and elongated face). Two other signs of pathology were noted but not scored for in this experiment: asymmetry and embryo body opacity.

A previous study by Foran *et al.* (2004) exposed adult Japanese medaka to FLX at aqueous nominal doses between 0.1 and 5.0 µg/L for 4 weeks, with reproductive assessments conducted during the last 2 weeks. Reproductive assessments evaluated endpoints on the number of eggs produced, mean number of eggs per day, percentage of days on which spawning occurred, percentage of eggs fertilized, percentage of fertilized eggs that hatched, and percentage of hatchlings with developmental abnormalities.

Developmental abnormalities present in the embryos included edema, curved spine, incomplete development (no pectoral fins, reduced eye size), and non-responsiveness to disturbances. Thus, the abnormalities noted in this experiment were quite similar, but there was a disparity between the two studies due to a difference in experimental design.

In addition, no model of toxicity was proposed for the embryotoxicity observed in the experiment by Foran et al. (2004). As developmental abnormalities in the embryos of exposed adults could be explained by different models of toxicity, two were considered in this study: (1) FLX acts as a mutagen to the germ cells of adults to cause embryotoxicity or (2) FLX is maternally-transferred to embryos during oogenesis at toxic doses. The present study tested the maternal transfer of FLX by applying topical dosages directly onto the surface of medaka eggs. This direct exposure may have generated additional signs of developmental abnormalities that were not previously observed by Foran et al. (2004).

Conversion of the doses (0.1 to 5.0 $\mu\text{g/L}$) tested by Foran et al. (2004) can be used to estimate the dose of FLX in medaka eggs by using a bioconcentration factor (BCF) of 74 for FLX (Paterson and Metcalfe, 2008). Calculations using the BCF, approximated to 100, yielded an estimated FLX tissue dose range of 1.0×10^{-5} to 5.0×10^{-4} $\mu\text{g/mg}$ in medaka eggs (similar calculations in Appendix 5). This is interesting to note as a broader range of doses (1.0×10^{-6} to 10 $\mu\text{g/mg}$) was tested in this study, which may have further contributed to additional signs of pathology observed in all FLX treatments. In addition, Foran et al. (2004) found the percentage of abnormal embryos to be between 1.97% to 2.53% for all tested concentrations. At the concentrations in medaka eggs (1.0×10^{-5} to 5.0×10^{-4} $\mu\text{g/mg}$) tested by Foran et al. (2004), the 30 minute exposure results (Figure 4) estimated the percentage of abnormal embryos to be between 0 to 20%. Therefore, a 2 to 10-fold increase

in the percentage of abnormal embryos was observed in this experiment compared to Foran et al. (2004). The discrepancy in percentage of abnormal fish is likely due to differences in experimental designs. In this study, FLX was directly applied onto the surface of medaka eggs, which may have contributed to the higher percentage of abnormal hatchlings.

In addition, the low percentage of abnormal embryos determined by Foran et al. (2004) may not represent toxic responses in embryos of exposed adults, but may be baseline frequencies of abnormalities observed in embryos of Japanese medaka as seen by the low percentage (5%) of abnormal embryos in the ERS control for the 30 minute FLX exposure. If this is the case, the results by Foran et al. (2004) show that a toxic dosage was not reached at the tested concentrations to generate high levels of embryotoxic response for the maternal transfer of FLX.

Further studies are required to provide greater insight on the mechanism of action and physico-chemical properties of FLX in generating developmental abnormalities in embryos. One such study could use radioisotopes to label FLX and then chromatographic separation to determine whether FLX is present in the lipids or germ cells of adult medakas. Information from using radioisotopes to label FLX would allow for better determination of the mechanism of action in which FLX can affect embryos.

Decreased Percent Normal from Fluoxetine Exposure

A strong negative linear relationship ($r^2 = 0.76$, $p = 0.005$) was observed in the 1 hour exposure between the percent of normal fish and the dose of FLX. However, the 1 hour exposure caused mortality in over 50% of the eggs for each FLX treatment soon after exposure. Even the lowest FLX dose (1.0×10^{-6} $\mu\text{g}/\text{mg}$) had elevated percent mortality and generated abnormalities in over 50% of embryos. In addition, no correlation ($p > 0.05$) was

observed between the percent mortality and dose of FLX. This suggests that signs of abnormalities may have been caused by severe stress from the long duration of exposure. Hence, these results did not provide useful information on the toxicity of FLX.

The 30 minute exposure yielded a strong negative linear relationship ($r^2 = 0.61$, $p = 0.022$) between the percent of normal fish and the dose of FLX. Basal levels of percent mortality were observed up to the threshold at $0.1 \mu\text{g}/\text{mg}$, and a 20% mortality was observed at higher doses of 1 and $10 \mu\text{g}/\text{mg}$. As a 30 minute exposure did not appear to induce severe stress on the embryos, these results provided better information on embryotoxicity.

Compared to other signs of pathology, the most severe response was observed for body hemorrhaging (BH) (prevalence range of 0 to 75%) (Table 2). BH was estimated to have an ED50 of $3.0 \times 10^{-3} \mu\text{g}/\text{mg}$, while other signs of pathology had ED50s $> 10 \mu\text{g}/\text{mg}$. An inverse relationship was also observed only between the prevalence of BH and percent normal, compared to other signs of pathology, after a 30 minute exposure. This is important as the percent normal (Figure 4) includes results for all signs of pathology, which suggests that BH is the main contributor to the decreased percentage of normal embryos. In addition, a moderate prevalence (0 to 50%) of embryo body opacity (EBO) was observed throughout the FLX treatments, though it was not scored for embryotoxicity. The high prevalence for BH and EBO, compared to other signs of pathology, was not observed by Foran et al. (2004). The differences in results and experiment design between Foran et al. (2004) and this present study suggests that a direct comparison between the two studies may not be drawn as there are differences in embryotoxicity responses. However, as topical doses of FLX generated signs of pathology in embryos, it suggests

that if FLX is maternally-transferred to oocytes during sexual maturation it can cause developmental abnormalities in embryos.

Variability was also observed in the data points for percent normal along the linear regression line (Figure 4), in which an increase in the percentage of normal embryos was observed at the two highest doses (1 and 10 $\mu\text{g}/\text{mg}$). The variability in percent normal may be explained using the uptake kinetics theory. This theory suggests that after all receptors on the presynaptic membrane are bound to FLX, inhibiting the binding of serotonin to reuptake receptors, an increase in FLX dose will not cause an additional effect as a threshold limit has already been reached. In addition, variability in percent normal may also be explained by random variation due to baseline abnormalities in Japanese medaka as different fish organisms may generate variable differences in baseline abnormalities.

Therefore, the results from this study suggest that FLX maternally-transferred could cause embryotoxicity as seen from the decrease in percent normal (Figure 4). It was determined that this toxicity is mainly contributed by the increase in prevalence of body hemorrhaging. Thus, this model of toxicity, testing the maternal transfer of FLX to embryos of exposed adults, cannot be rejected. However, future research to look at quantifying the internalized doses of FLX in medaka eggs is needed to confirm that FLX may be maternally-transferred to cause embryotoxicity.

Research to look at the alternative model of toxicity, whether FLX causes mutation in offspring by acting directly on the germ cells of adults, is also needed to help in understanding of the mechanism of action for FLX. Previous research on FLX has shown that it does not exhibit mutagenic activity in vitro in mammalian cell or microbial test systems, though no research has been conducted on fish models (Lilly, 2005). One method

for mutagenicity testing in fish is the measurement of DNA fragmentation. Mutation on the DNA recognition sites is known to alter the ability of restriction enzymes to cut the DNA at specific sites (Tomic *et al.*, 1990). Thus, if FLX acts as a mutagen, it will produce variable fragment lengths of DNA instead of the expected fragment lengths.

Environmental Significance

As the quantity of antidepressants entering into aquatic environments increases, information on possible maternal transfer of FLX to fish will be of considerable ecological interest. Recently, Kolpin *et al.* (2002) detected FLX concentrations at 0.012 µg/L in streams in the United States between 1999 to 2000, and Metcalfe *et al.* (2003b) detected FLX concentrations between 0.013 to 0.099 µg/L in sewage treatment plant effluents near the Great Lakes, Canada. Notably, Weston *et al.* (2001) detected effluent FLX concentrations as high as 0.540 µg/L. If these FLX concentrations are found in medaka embryos, the results from this study suggest that an elevated incidence of developmental abnormalities and mortalities will be observed. Thus, determining how this drug and its metabolites affect embryo development will help researchers and scientists anticipate how these compounds will impact the environment.

Further research is needed to determine how bioaccumulative and persistent FLX is in the environment. In addition, conducting research for other antidepressant pharmaceuticals and their effects on aquatic organisms will also be beneficial as these compounds may generate different signs of pathology to embryos. Once in the environment, antidepressants are rarely present as single compounds, but rather as components of complex mixtures. Previous findings have demonstrated that certain SSRI mixtures exhibit additive effects on various organisms in the environment (Oakes *et al.*, 2010). Thus, more

research is required to learn the mechanisms of how individual antidepressants work on aquatic organisms to understand how complex mixtures of antidepressants may react in the environment.

Conclusion

This study's objective was to assess a model of exposure and toxicity by which FLX may generate signs of pathology in the offspring of exposed adults. The results indicated that if the FLX accumulated by adult medaka is transferred to oocytes during oogenesis, it could represent a significant source of toxicity by generating signs of pathology in hatchlings. Research to characterize the actual dose received in the medaka eggs from maternal transfer will provide information on whether the maternal transfer of FLX can cause embryotoxicity. Future studies must be conducted to assess the alternative model of toxicity to determine whether FLX may also act as a mutagen to the germ cells of adults in causing signs of pathology in embryos.

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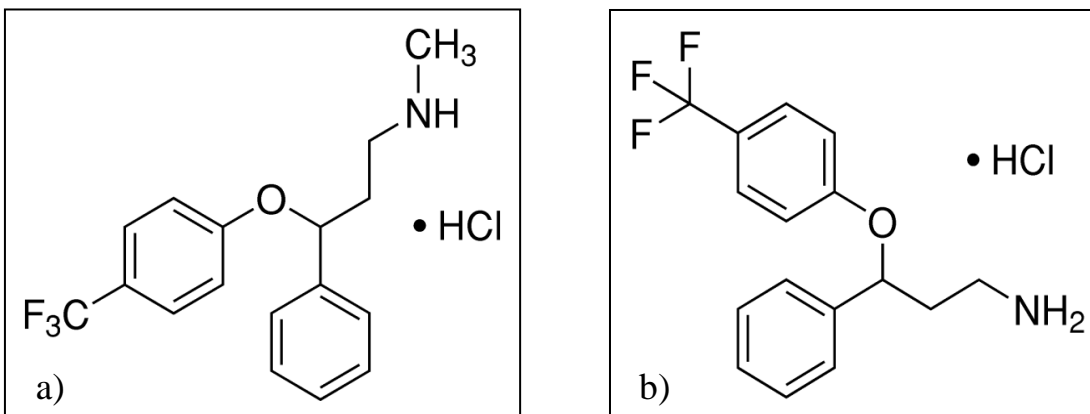
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SUMMARY

1. The waterborne exposure and nano-injection method were not suitable for modelling the maternal transfer of fluoxetine (FLX).
2. Topical application was a practical method to mimic maternal transfer.
3. The 1 hour FLX exposure caused that abnormalities and severe stress. These results did not provide information on the toxicity of fluoxetine exposure.
4. A strong negative linear relationship between the percent normal and dose of FLX was observed for the 30 minute exposure. This decrease in percent normal was mostly contributed by a high prevalence of body hemorrhaging (BH) compared to other signs of pathology.
5. Basal levels of mortality were observed in all FLX doses, except at 1 and 10 $\mu\text{g}/\text{mg}$ where 20% mortality was observed.
6. The ED50 for BH was $3.0 \times 10^{-3} \mu\text{g}/\text{mg}$, while the ED50s for all other signs of pathology were $> 10 \mu\text{g}/\text{mg}$.
7. Asymmetry and embryo body opacity were also observed in medaka embryos from FLX exposure, although these signs were not incorporated in embryotoxicity scoring.
8. FLX may be maternally-transferred to oocytes via lipids during sexual maturation. However, research to quantify the internalized dose of FLX in medaka eggs are needed to provide more information on the maternal transfer of FLX.
9. Future studies of alternative models of toxicity are needed to determine whether fluoxetine may also act as a mutagen on the germ cells of adults.

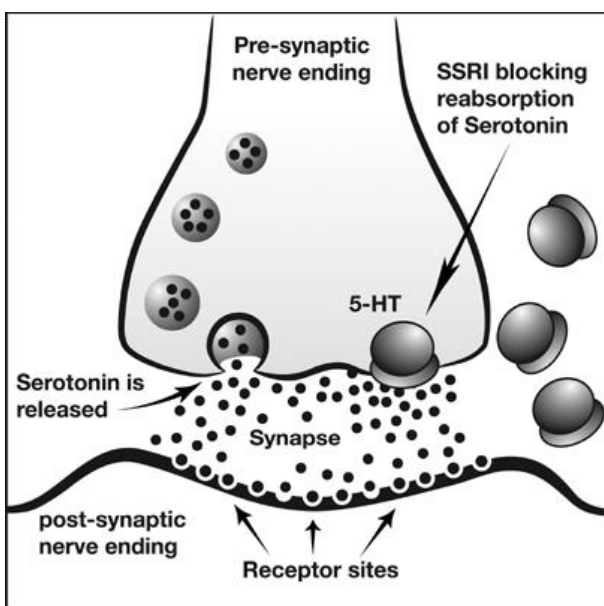
APPENDICES



Appendix 1: Chemical structure of (a) fluoxetine hydrochloride and (b) norfluoxetine hydrochloride (Sigma-Aldrich, retrieved from (a)

http://www.sigmaaldrich.com/catalog/ProductDetail.do?D7=0&N5=SEARCH_CONCAT_PNO%7CBRAND_KEY&N4=F132%7CSIGMA&N25=0&QS=ON&F=SPEC and (b)

http://www.sigmaaldrich.com/catalog/ProductDetail.do?D7=0&N5=SEARCH_CONCAT_PNO%7CBRAND_KEY&N4=F133%7CSIGMA&N25=0&QS=ON&F=SPEC).



Appendix 2: Mechanism of SSRI (Science Blogs, retrieved from

<http://scienceblogs.com/neurotopia/SSRI.jpg>)

Appendix 3: Preparation of Embryo Rearing Solution (ERS) in stock aliquots

Embryo rearing solution was prepared by making stocks of four salts, and adding the stocks together. This solution was enough to make nine 50 mL aliquots of 20 times concentrated stock (9 L total of rearing solution). The following four stocks were prepared by adding the proper amount of each salt to 100 mL of deionized water in an Erlenmeyer flask. A stir bar was used to spin the salt into solution.

<u>Stock</u>	<u>Preparation</u>
1. 10% NaCl:	10 g NaCl in 100 mL dH ₂ O
2. 0.4% CaCl ₂ 2 H ₂ O:	0.4 g CaCl ₂ 2 H ₂ O in 100 mL dH ₂ O
3. 0.3% KCl:	0.3 g KCl in 100 mL dH ₂ O
4. 1.63% MgSO ₄ 7 H ₂ O:	1.63 g MgSO ₄ 7 H ₂ O in 100 mL dH ₂ O

The above 100 mL solutions were added to a 500 mL Erlenmeyer flask, an extra 100 mL of dH₂O were added (total volume should be 500 mL). The stock solution was split into nine 50 mL aliquots using a graduated cylinder and stored in conical test tubes at 4°C. When ready to be used, a test tube of concentrated ERS was removed from the fridge and left out over night to bring the solution up to room temperature. The 50 mL concentrated ERS solution was then poured into a 1 L flask and 950 mL dechlorinated water and one drop of methylene blue mold inhibitor was added (Modified from Glase and Reed, 2000).

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Appendix 4: Needle Filling for Nano-Injections

An Eppendorf microloader was placed on the end of a pipette, and filled to 8 μl . The fine tip of the microloader was threaded into the open or back end of the glass injection needle all the way to its constriction. When the microloader tip was fully inserted, the dosing solution was dispensed into the injection needle, making sure not to produce any bubbles. The needles were secured on a strip of plasticine clay in a square Petri dish and stored at room temperature.

Appendix 5: Developing Range of Exposure Doses

A) Bioconcentration factor (BCF) of FLX = $\frac{\text{Concentration in tissue}}{\text{Concentration in water}} = 74$

(Paterson and Metcalfe, 2008) = rounded up to 100

B) Previous studies by Schultz et al. (2010) showed toxic effect observed when the concentration of FLX in water = 1 $\mu\text{g/L}$

C) Estimated dose of fluoxetine in fish tissue:

$$= 1 \mu\text{g/L} \times 100 \text{ (BCF)}$$

$$= 100 \mu\text{g/L}$$

$$= 100 \mu\text{g/kg of fish (assuming water density} = 1.0 \text{ L/kg)}$$

$$= 0.1 \mu\text{g/g of fish}$$

D) Since 1 medaka egg = approximately 1 mg

$$= 0.0001 \mu\text{g/mg (toxic effect seen in medaka egg)}$$

E) Testing Dose Range = 0.000001 $\mu\text{g/mg}$ to 10 $\mu\text{g/mg}$

Two assumptions were present for this calculation:

- 1) Dose of FLX in water (D_w) is at equilibrium to doses of FLX in the tissue of adult (D_T)
- 2) Dose of FLX in medaka egg is the same as dose of FLX in the body of the parent organism.