Waterborne fluoxetine exposure disrupts metabolism in *Carassius auratus*

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Abstract

Fluoxetine, a selective serotonin re-uptake inhibitor (SSRI) and the active ingredient in Prozac®, is found in the environment and disrupts feeding and metabolism in exposed fish. The objective of this research was to investigate the mechanisms involved in the feeding and metabolism disruption in the model goldfish (*Carassius auratus).* Two short-term waterborne fluoxetine exposures (7- and 14-days) were performed using two environmentally relevant doses of fluoxetine (0.5 and 1 μg/L) and metabolic effects at the level of the brain, liver, serum and bile in goldfish were investigated. Abundances of mRNA transcripts coding for six feeding neuropeptides were examined to determine which may be involved in the initial neural changes associated with decreased appetite in goldfish. The 7-day fluoxetine exposure at 1 μg/L caused corticotropin-releasing factor (CRF) mRNA levels to increase by 2-fold in female hypothalamus and telencephalon, indicating that CRF may be one of the first of the feeding neuropeptides to be altered. Six hepatic miRNAs were also evaluated in the goldfish liver that were previously associated with fluoxetine exposure in zebrafish (*Danio rerio*). Following the 7-day exposure at 1 μg/L, miR-22b, miR-140, miR-210, miR-301a and miR-457b levels increased in the female goldfish liver by 4-6 fold. The 14-day fluoxetine exposure at 1 μg/L caused 2-fold increases in miR-210, miR-301a, miR-457b and let-7d in male goldfish liver. These miRNAs were associated with the down-regulation of anabolic metabolic pathways in zebrafish, indicating a conservation of miRNA and fluoxetine effect between fish species. Serum and bile metabolite profiles of fluoxetine exposed goldfish were evaluated using ultra performance liquid chromatography coupled to quadrupole time of flight mass spectrometry. Following the 14-day exposure at 1 μg/L, the bile metabolite profiles of male goldfish were significantly different from controls as detected by cluster analysis and fluoxetine was tentatively identified in the serum. No other discriminant metabolites were identified as of yet. The data presented suggest that fluoxetine causes metabolic disruption in goldfish at multiple organ levels. Because of the widespread detection of fluoxetine and other emerging SSRIs in the aquatic environment, future research is required to firmly establish this pharmaceutical class as a metabolic and endocrine disrupting chemical.

Résumé

La fluoxétine, l'ingrédient actif du Prozac®, est un inhibiteur sélectif de la recapture de la sérotonine (ISRS). Cet inhibiteur est retrouvé dans l'environnement et perturbe l'alimentation et le métabolisme chez les poissons. Cette recherche a pour but de comprendre et d'étudier les mécanismes qui sont impliqués dans la pertubation de l'alimentation et le métabolisme chez le poisson rouge (*Carassius auratus*). Les effets métaboliques de la fluoxétine dans le cerveau, le foie, le sérum et la bile des poissons rouges ont été étudiés au cours de deux études à court terme (7 et 14 jours) à une exposition à la fluoxétine dans l'eau à deux concentrations pertinentes de l'environement (0.5 et 1 µg/L). Des analyses de plusieurs ARNm qui codent pour six neuropeptides qui régularisent l'alimentation ont été effectuées pour déterminer si ceux-ci sont impliqués dans les changements neuronaux initiales liées à une dimunition de l'appétit chez le poisson rouge. Les poissons rouges qui ont été exposés à une concentration de fluoxétine de 1ug/L pendant 7 jours révèlent que l'ARNm du facteur de relâche de la corticotropine (CRF) augmente de 3 fois dans l'hypothalamus et le télencéphale chez la femelle du poisson. Ce résultat indique que le facteur CRF peut être l'un des premiers neuropeptides à être modifié. De plus, six miARN hépatiques ont été précédemment associé à l'exposition fluoxétine chez le poisson-zèbre (*Danio rerio*) donc ceux-ci ont été également étudié dans le foie des poissons rouges. Plus précisement, le niveau de miR-22b, miR-140, miR-210, miR-301a et miR-457b ont augmenté de 4 à 6 fois dans le foie des poissons rouges femme suite à l'exposition fluoxétine de 7 jours à une concentration de 1ug/L. De plus, une augmentation de 2 fois est observée chez les miARN nommés miR-210, miR-301a, miR-457b et let-7d dans le foie des poissons rouges mâle suite à l'exposition fluoxétine de 14 jours à une concentration de 1 µg/L. Les résultats suggèrent qu'il y a un niveau de conservation entre les miARN et les effets de la fluoxétine chez les poissons car ces miARNs exercent une régulation négative sur les voies du métabolisme anabolisants chez le poisson zèbre. Le spectromètre de masse hybride quadripôle-temps de vol utilisé en couplage avec la chromatographie en phase liquide a été employé pour accomplir l'analyse de profils métaboliques dans le sérum et la bile des poissons rouges exposés à la fluoxétine. La fluoxétine a été tentativement identifiée dans la bile et on retrouve une différence significative chez les mâles entre les poissons rouges contrôles et les poissons rouges exposés à la fluoxétine d'une durée de 14 jours à une concentration de 1 µg/L suite à l'analyse de grappe. À la suite de cette étude, aucun autre métabolite discriminant a été identifié jusqu'à maintenant. Les données suggèrent que la fluoxétine provoque une perturbation métabolique au niveau de plusieurs organes. Des recherches futures sont nécessaires pour établir cette classe pharmaceutique comme un produit chimique pertubateurs des systèmes de métabolisme et d'endocrinien car la fluoxétine et d'autres ISRS sont retrouvés en abondance dans l'environnement.

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Abbreviations

17,20p	17a,20P-Dihydroxy-4-pregnen-3-o
5-HT	5-hydroxytryptamine (serotonin)
AAs	amino acids
ACTH	adrenocorticotropic hormone
AMPK	AMP-activated protein kinase
ACN	acetonitrile
BCF	bioconcentration factor
CART-1	cocaine- and amphetamine- related transcript-1
CCK	cholecystokinin
cDNA	complimentary DNA
CE	capillary electrophoresis
CRF	corticotropin-releasing factor
CYP	cytochrome
DNA	deoxyribonucleic acid
EDC	endocrine disrupting chemical
ELISA	enzyme-linked immunosorbent assay
ESI	electron spray ionization
FK506	fujimycin
FSH	follicle stimulating hormone
FSH	follicle stimulating hormone receptor
GABA	gamma-aminobutyric acid
GC	gas chromatography
GSI	gonadal somatic index
HILIC	hydrophilic interaction chromatography
HP	high performance
HPI	hypothalamic pituitary interrenal
HSI	hepatosomatic index
IST	isotocin
LC	liquid chromatography
LOEC	lowest observable effect concentration
miRNA	micro RNA
mRNA	messenger RNA
MS	mass spectrometry
MS-222	3-aminobenzoic acid ethylester
NMR	nuclear magnetic resonance
NPY	neuropeptide Y
NT	novel tank
OPLS-DA	orthogonal partial least square- discriminate analysis
PBDE	Polybrominated diphenyl ethers
PCA	principle component analysis
PCB	Polychlorinated biphenyls
PG	prostaglandin

PGF2α	prostaglandin f2alpha
PI3K	phosphoinositide 3-kinase
Pre-miRNA	precursor micro RNA
Pri-miRNA	primary micro RNA
PRL	prolactin
Q-TOF	quradrupole time of flight
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RT-PCR	real-time polymerase chain reaction
SERT	serotonin re-uptake transporter
SGa	secretogranin-IIa
SNa	secretoneurin-a
SSRI	selective serotonin reuptake inhibitor
T	testosterone
TCDD	2,3,7,8-tetrachlorodibenzo- p-dioxin
TH	thyroid hormone
UP	ultraperformance
URC	urocortin
UTR	untranslated region
VTG	vitellogenin

CHAPTER 1:

Introduction

1.1 Outline and rationale

Fluoxetine is the active ingredient in Prozac®, a selective serotonin reuptake inhibitor (SSRI) used to treat depression. Due to its heavy prescription use, incomplete metabolism in humans and inefficient removal from wastewater, fluoxetine is detectable in aquatic environments downstream of wastewater treatment plants[1]. Fluoxetine is known to disrupt several endocrine systems of fish by acting on the serotoninergic pathways, thus altering biological systems controlled by serotonin such as feeding, metabolism and reproduction [2–4]. Currently, little is known about the mechanisms behind many of these disturbances, specifically fluoxetine-induced metabolic changes. The aim of this research was therefore to uncover potential mechanisms for the metabolic upset caused by fluoxetine exposure in a teleost fish. To accomplish this goal, the goldfish (*Carassius auratus)* model was used in two short-term waterborne fluoxetine exposures. To determine the effects of fluoxetine exposure that may lead to metabolic changes, we examined brain mRNA levels, hepatic miRNA levels and small molecule signal intensities in serum and bile.

Messenger RNA (mRNA) of neuropeptides that regulate food intake were compared in the hypothalamus and telencephalon of goldfish following a 7-day fluoxetine exposure to determine any neurological effects associated previously observed changes in fish feeding behaviour (goldfish, striped bass)[2,5]. By examining the differences in expression of feeding neuropeptides caused by fluoxetine exposure, metabolic upset could be considered in the context of disrupted feeding pathways in the brain. Secondly, hepatic microRNAs (miRNAs) were evaluated following previous research by Craig et al.[6],which suggested that a potential

mechanism of fluoxetine-induced metabolic upset in zebrafish (*Danio rerio)* is driven by miRNA. The levels of six hepatic miRNAs were investigated in goldfish following two short-term waterborne exposures to see if there is a conservation of effect between fluoxetine and miRNA and to confirm the role of miRNA as regulatory molecules in fluoxetine-induced metabolism upset. Finally, the serum and bile metabolite profiles of fluoxetine-exposed goldfish were compared to those of unexposed fish to determine any major metabolite changes using accurate mass spectrometry-based non-targeted metabolomics. Any discriminant metabolites found in exposed fish may reveal downstream effects of modified metabolic activity and fluoxetine-affected pathways could be proposed in a bottom-up approach.

Through the assessment of feeding neuropeptides, hepatic miRNA and serum and bile metabolite profiles, a systems approach was taken to understand the mechanisms behind fluoxetine-induced metabolism disruption. For this research, the primary hypotheses and predictions were:

(1) Waterborne fluoxetine exposure upsets feeding neuropeptides in goldfish brain

Predictions: Anorexigenic neuropeptide mRNA levels (corticotropin-releasing factor (CRF), cholecystokinin (CCK), and cocaine- and amphetamine- related transcript-1 (CART-1) and isotocin) will increase following fluoxetine exposure, while orexigenic neuropeptide levels (neuropeptide Y (NPY), orexin-A, and secretogranin-IIa (SGIIa) will decrease (Chapter 2).

(2) Waterborne fluoxetine exposure increases hepatic miRNA in goldfish

Predictions: Hepatic transcript abundance of *dre-miR-22b, dre-miR-140, dre-miR-210a, dre-mir-301, dre-miR-457b,* and *dre-let-7d* will increase following fluoxetine exposure (Chapter 2).

(3) Null hypothesis: there will be no differences between the serum and bile metabolite profiles of fluoxetine exposed goldfish compared to control goldfish (Chapter 3).

1.2. Goldfish as a model for the analysis of neuroendocrine disruption

Goldfish are part of the family *Cyprinidae*, one of the largest vertebrate families. This family contains over 2,000 species including carps and minnows, which are native to North America and may be found in potentially fluoxetine-exposed waters. Fluoxetine has been detected in the brain of wild cyprinid species, including the common carp (*Cyprinus carpio*), therefore the effects of fluoxetine on goldfish brain and other tissues will be especially applicable[7]. Goldfish are also useful models in endocrine disruption research due to the extensive understanding of their neuroendocrine systems, specifically the serotoninergic pathway (see section 1.3). Finally, goldfish are relatively easy to house and handle and are large enough to collect sufficient quantities of tissue for extracting DNA, RNA, proteins and metabolites. As such, goldfish are an excellent model for the purpose of this research.

1.3. Serotonin and the feeding system in teleost fish

Fluoxetine alters levels of serotonin (5-hydroxytryptamine, 5-HT) in vertebrates by blocking the re-uptake of 5-HT in serotoninergic cells. Serotonin is an evolutionarily ancient amide found across both the plant and animal kingdoms[8]. In vertebrates, 5-HT is a signalling molecule and its receptors are found in a variety of tissues including brain, liver, kidney, testes and lung, with the majority of 5-HT produced in the gut[9]. The key enzyme involved in 5-HT synthesis is tryptophan hydroxylase, which converts the essential amino acid L-tryptophan into 5-hydroxytryptophan[10]. The enzyme amino acid decarboxylase then converts 5-hydroxytryptophan into 5-HT, where it is stored in synaptic vesicles and released into the synapse as a result of neural membrane depolarization.

Once in the synapse, 5-HT binds to target cell 5-HT receptors. Nearly all 5-HT receptors are G-protein coupled, with the exception of the ligand-gated ion channel 5-HT_3 receptor. The seven trans-membrane domain 5-HT receptors activate upon binding with the extracellular 5-HT and begin a cascade of effects downstream in the target cell. 5-HT activity is then terminated by its re-uptake into the pre-synaptic cell via a serotonin re-uptake transporter (SERT), the target of fluoxetine.

The general organization of the 5-HT systems in teleost brain is somewhat similar to that of mammals, with fibres extending into the telencephalon, hypothalamus, optic tectum, medulla and the pars distalis of the pituitary gland[11]. While 5-HT is known to influence the reproductive and growth systems in goldfish[12], its impact on the neural input in feeding and satiety signals is not as well understood. Briefly, food intake in goldfish is regulated by orexigenic (appetite inducing) and anorexigenic (appetite inhibiting) signals. In the hypothalamus of the brain, neuropeptide Y (NPY) is the most potent orexigenic factor involved in feeding behaviour in fish and increases following a period of fasting[13]. Serotonin is known to inhibit NPY activity in the brain and thus acts as an anorexigenic neurotransmitter[14]. Other orexigenic neuropeptides in fish are orexin A and galanin, which interact with NPY in a coordinated circuit system to induce feeding (see Fig. 1.1).

Anorexigenic peptides inhibit feeding and include cholecystokinin (CCK), cocaine and amphetamine- regulated transcript-1 (CART-1) and corticotropin-releasing factor (CRF), amongst others. CCK and CART-1 negatively regulate NPY and the orexins in the hypothalamus and their levels decrease following food deprivation[15]. Serotonin is known to inhibit feeding when injected into fish brains and its anorexigenic effects are partially mediated through at least one of these anorexigenic peptides, CRF[16].

Other feeding neuropeptides are also involved in this complex circuit, such as bombesin, tachykinins, somatostatin and urotensin, but the effects of 5-HT on these neuropeptides are not understood. In summary, 5-HT stimulates neuropeptides in the brain that act to decrease feeding and induce satiety such as CRF, while inhibiting neuropeptides that promote feeding, such as NPY. If an endocrine disrupting chemical such as fluoxetine alters 5-HT levels in the brain, these neuropeptides are also likely to be altered and feeding motivation and behaviour will be disrupted.

1.4. Fluoxetine as an endocrine disrupting chemical

Antidepressants are psychoactive pharmaceuticals and are prescribed to treat a variety of psychological distresses such as depression, anxiety, bulimia and obsessive compulsive disorder. Antidepressants are one of the most commonly prescribed drugs in North America and in 2011, were being taken by over 10% of the North American (USA) population[17]. These drugs are frequently prescribed long-term, with 60% of patients taking these pharmaceuticals for over 2 years. With such abundant and persistent use, antidepressants have become potential endocrine disrupting chemicals (EDCs) as they enter the environment following improper disposal and inadequate metabolism and removal from wastewater treatment plants. Antidepressants are being reported in concentrations up to several ng-µg/L in raw sewage, effluent from sewer treatment facilities, rivers downstream of such facilities and have been detected in drinking water in trace levels[18–20].

Prozac® is one of the most prescribed antidepressants and its active ingredient fluoxetine is not fully metabolised in the human liver when administered. Approximately 11% of ingested fluoxetine is excreted as the parent compound while 7% is excreted as the equally potent de-methylated metabolite, norfluoxetine [21]. Fluoxetine is resistant to hydrolysis and photolysis and

has been found in the aquatic environment following inefficient waste water treatment in concentrations ranging globally from 0.012 to 1.4 μg/L[22–26]. Research on the biological effects caused by environmental fluoxetine exposure is therefore essential to better understand the extent of exposure effects of this EDC.

1.4.1. Fluoxetine properties

Fluoxetine (Fig.1.3) is an SSRI and works by blocking the re-uptake of 5-HT into serotoninergic neuronal terminals. Specifically, fluoxetine binds to and inhibits the 5-HT re-uptake transporter (SERT) *slc6a4*. This transporter is found in serotonergic neurons in the teleost brain[27] and is widely expressed in goldfish tissues such as testis, liver and gut[3]. When SERT is blocked, the 5-HT signal to the post-synaptic cell is prolonged, ultimately resulting in an up-regulation of the 5-HT-dependent circuits (see Fig 1.2).

Fluoxetine is relatively lipophilic and therefore has a high volume of distribution and can easily pass through the blood brain barrier once administered[28]. Fluoxetine is also a small molecule, with a molecular mass of 309.33 g/mol and while relatively hydrophobic, with a logKow (Octanol-Water Partition Coefficient) of 3.98, is still soluble in water (5mg/mL). Fluoxetine is a weak base, with a pKa of 10.1 and therefore ionizes at pHs relevant to freshwater aquatic environments. In mammals, fluoxetine is metabolized by cytochrome (CYP) P4502D6 and its major metabolite is the equally potent norfluoxetine. Fluoxetine and nor-fluoxetine are both chiral molecules (see Fig. 1.3). The S-enantiomers of each molecule are more potent in the inhibition of 5-HT reuptake and remain longer in the plasma than the R-enantiomers in fathead minnows and striped bass[29,30].

In several fish species, however, CYP is not the key enzyme for hepatic metabolism of fluoxetine[4]. While still found in fluoxetine-exposed fish tissues, norfluoxetine is not the

predominant biotransformation metabolite in fathead minnows (*Pimephales promelas)*[31]. The main metabolism process of fluoxetine in teleost fish is still unknown. The half life of fluoxetine in Japanese medaka *(Oryzias latipes*) is 9 days[30], which is three times that of a mammal due to the lower biotransformation and fluoxetine elimination in fish. Fluoxetine was calculated to have a bioconcentration factor (BCF) in Japanese medaka in the range of 74-80, therefore, fluoxetine has the ability to bioaccumulate and bioconcentrate in fish tissues.

1.4.2. Effects of fluoxetine on fish physiology and behaviour

Fluoxetine is administered to improve mood by altering 5-HT response in the brain, but the antidepressant often has serious side effects in humans associated with other 5-HT related endocrine systems. Patients who take fluoxetine often experience sexual dysfunction, weight gain, nausea and sleep disturbances[32]. The 5-HT neurotransmission system is highly conserved across vertebrates and therefore negative non-target hormonal effects have also been observed in exposed animals. Fluoxetine has been shown to cause neuroendocrine disruption in feeding, reproduction and behaviour on non-target species due to its effect on 5-HT pathways[3,5,33].

Waterborne fluoxetine exposure upsets reproductive hormones such as estradiol and testosterone in teleost fish, though the findings are not consistent across species, tissues and fluoxetine administration[3,34–38]. In adult male goldfish, a 14-day waterborne exposure of 0.54 μg/L fluoxetine decreased circulating testosterone levels and increased circulating estradiol [3]. A 28-day exposure of similar fluoxetine concentrations also increased circulating estradiol levels in female Japanese medaka[34]. However, levels of testosterone and estradiol in fathead minnows were unaffected following a 28-day exposure at 0.1-100 μg/L [35] and at 32 μg/L, a 7-day exposure decreased ovarian estradiol levels in zebrafish[36]. An injection study similarly found that five injections of fluoxetine over 14 days in female goldfish reduced plasma estradiol levels by

75% [37]. Based on these conflicting findings in the literature, the relationship between fluoxetine and the reproductive hormones is metabolically complex and requires further investigation to explain the observed differences.

Gonad physiology is also impacted following fluoxetine exposure, with varying results across species and treatment[3,31,34,36,38]. Milt volume decreased and female pheromone-induced milt release was inhibited in male goldfish following a 14-day exposure to 54 μg/L and 0.54 μg/L fluoxetine, respectively, although gonad histology remained normal[3]. A 7-day exposure of 32 μg/L fluoxetine decreased zebrafish egg production[36]. Male fathead minnows exposed to lower levels of fluoxetine (0.028 μg/L) had induced vitellogenin in plasma, as well as testicular interstitial cell prominence following short-term exposure[31]. No changes were observed, however, in fecundity, fertility, rate of spawning or hatching success in Japanese medaka exposed to fluoxetine for 28 days (0.5-5 μg/L)[34]. Mating behaviours are also disrupted by fluoxetine exposure. Siamese fighting fish (*Betta splendens*) reduce aggressive display after fluoxetine exposure of 0.54 μg/L for 5 hours[38]. Male fathead minnows also increased nest cleaning, defending and decrease mating behaviour following 28 days of 1-100 μg/L fluoxetine exposure [35]. Fluoxetine ultimately decreases reproductive output in exposed fish through modified reproductive hormones and behaviours.

Fish feeding and metabolism are also impacted following fluoxetine exposure, though fewer studies have addressed this system[2,5,29,37]. A 28-day exposure to waterborne fluoxetine at environmentally relevant concentrations (0.54 μg/L) disrupted feeding and energy metabolism in goldfish, down-regulating hepatic gluconeogenesis and up-regulating glycolysis through hexokinase and glucokinase activity[5]. Fluoxetine exposure also reduced feeding frequency in striped bass (*Morone saxatilis*×*M. chrysops*)[2] and fathead minnows[29] following 7-day fluoxetine

exposures (35 μg/L and 50 μg/L, respectively). Few studies have investigated the mechanisms behind these feeding disruptions. Mennigen et al[5] considered the effect of fluoxetine on the transcript abundance of several feeding neuropeptide levels and found that at low doses, fluoxetine significantly reduced transcript levels of orexigenic NPY in the hypothalamus, while at higher doses, fluoxetine exposure increased NPY mRNA. Wong et al. found similar increases in NPY mRNA levels in male zebrafish brain following a high fluoxetine exposure[39], however, these results should be interpreted cautiously as whole brain levels were studied, thus no detailed tissue function can be extrapolated. As with the effect of fluoxetine on reproductive hormones, the impact of fluoxetine on brain chemistry is still unclear.

Other fluoxetine exposure studies have focussed on the stress axis of fish, where fluoxetine seems to have stress reduction effect in teleost fish at environmentally relevant and high doses[39–44]. Larval fathead minnows exposed to environmentally relevant levels of fluoxetine (0.025-0.25 μg/L) as embryos exhibited disrupted C-start performance, a predator avoidance mechanism, as well as slower escape velocity when compared to controls[40]. Chronic exposure to high doses of fluoxetine (14 days, 100 μg/L) reduced anxiety in male and female zebrafish as demonstrated by a novel tank test[41]. Zebrafish exposed to fluoxetine also had lower whole body cortisol levels when compared to controls following the 14-day experiment, in similarity to the decreased cortisol response by adult zebrafish seen by Sander de Abreu et al.[42]. A separate 14-day fluoxetine exposure to male zebrafish also caused stress reduction effects, as noted by similar novel tank observations and lower urocortin 3 levels in the brain[39]. Male fathead minnows increased exploratory behaviour following 14- and 28-day exposures to high doses of fluoxetine, though swimming speed was not affected [43]. A 96-hour fluoxetine exposure to zebrafish larvae decreased the expression of the gene coding for FK506 binding protein 5, a

protein annotated to stress response regulation, furthering the concept that fluoxetine is acting on the development of the stress response in larval fish[44]. From these studies, fluoxetine exhibits stress reducing effects at different doses and exposure times to various fish species.

Other behaviour effects have also been noted in fluoxetine-exposed fish[33,45,46]. Locomotor activity is reduced by fluoxetine exposure in as little as 56 hours in juvenile sheepshead minnow [45]. A 14-day exposure to 3 μg/L of fluoxetine lowered the aggression of Arabian killifish [33], while male Siamese fighting reduced aggressive display following a 6-day exposure to 0.54 μg/L fluoxetine [46]. These reductions in stress and aggression, in addition to the disruptions in reproductive and feeding physiology and behaviour, demonstrate the potential for fluoxetine to cause major population effects in aquatic ecosystems, most likely through its effect on 5-HT signalling in the teleost fish. The presented findings are outlined in Table 1.1.

Table 1.1 Waterborne fluoxetine exposures on various teleost fish models from 2004-2014.

Author, date	Ref	Animal	Nominal concentration (µg/L)	Calculated concentration (µg/L)	Duration	Effects
Foran et al., 2004	[34]	Male and female Japanese medaka (*Oryzias latipes)*	0.1, 0.5, 1 and 5	0.1 µg/L could not be detected, while 0.5, 1.0, and 5 µg/L were between 120 and 152% of nominal values	28 days	**0.1 and 0.5 µg/L**: ↑ plasma estradiol in females, embryo deformities **No effect**: fecundity, fertility, rate of spawning, hatching success, GSI, hepatic vitellogenin, and *ex vivo* gonadal steroidogenesis, circulating testosterone levels
Stanley et al., 2007	[29]	Juvenile fathead minnows (*Pimephales promelas)*	1, 10, 50, 100 and 250	89.7% ± 12.5% of nominal concentrations	7 days	**50 µg/L:** ↓in growth for S- and rac-fluoxetine, LOEC for feeding rate for S-fluoxetine
Gaworecki and Klaine, 2008	[2]	Male and female hybrid striped bass (*Morone saxatilis×M. chrysops)*	35, 75, and 150	23.2±6.6, 51.4±10.9, and 100.9±18.6	6 days followed by 6 day recovery	**All exposures:**↓ prey capture, whole brain 5-HT and 5-hydroxyindoleacetic acid levels
Egan et al., 2009	[41]	Male and female zebrafish (*Danio rerio)*	100	Not calculated	14 days	**100 µg/L:** ↓ latency to enter top half of novel tank, ↑ time spent in top half, ↑ transitions to top half. ↓ whole body cortisol levels **No effect:** velocity or distance traveled by fish
Lister et al., 2009	[36]	Male and female zebrafish (*Danio rerio)*	0.32, 3.2 and 32	Not calculated	7 days	**32 µg/L:** ↓ egg production, ovarian estradiol levels & testicular steroidogenesis, inhibited FSHr and LHr expression and aromatase in gonads

					No effect: GSI, PG synthesis, CYP expression in liver
Painter et al., 2009 [40]	Fathead minnow (*Pimephales promelas)* embryos and larvae	0.025, 0.125 and 0.25	Not calculated	5 days (embryo) or 12 days (larvae)	**0.25 µg/L**: ↓ escape velocity in embryos **No effect:** escape response following embryo exposure, escape behaviour after larval exposure
Mennigen et al., 2010a [3]	Male goldfish (*Carassius auratus)*	0.54 and 54	0.375±0.055 and 45±5.2	14 days	↓ pheromone mediated milt release and overall milt volume, T ↓ and estradiol ↑ in blood, FSH ↓ in pituitary and IST ↓ in telencephalon following 17,20p and PGF2α exposure **No effect:** weight, GSI
Mennigen et al., 2010b [5]	Female goldfish (*Carassius auratus)*	0.54 and 54	Not calculated	28 days	**0.54 µg/L:** ↓ circulating glucose, ↑ CRF and ↓ NPY in hypothalamus, ↓ of fructose-1,6-biphosphate in liver and ↓ in muscle hexokinase activity **54 µg/L:** ↓ in food intake and weight gain, ↑ cholesterol **Both**: Increased HSI **No effect**: glycogen levels, plasma protein and AAs
Schultz et al., 2011 [31]	Male fathead minnows (*Pimephales promelas)*	0.0025 and 0.028	0.0025±0.00099 and 0.028±0.0042	14 days	**28ng/L fluoxetine**: plasma VTG, testicular interstitial cell prominence **No effect:** GSI, HSI, spermatogonia or spermatozoa, hepatic adipocytes, vacuolization of hepatocytes, nest holding ability, detection in whole brain

Dzieweczynski and Hebert, 2012	[38]	Male Siamese fighting fish (*Betta splendens)*	0.54	Not calculated	5 hours	**0.54 µg/L:** ↓aggressive display **No effect:** Courting behaviour, time spent at nest
Park et al., 2012	[44]	Zebrafish (*Danio rerio)* larvae	25 and 250	Not calculated	96 hours	**25 µg/L**: 288 genes differentially expressed in whole body **250 µg/L**: 131 genes differentially expressed in whole body, FK506 binding protein down-regulated in both
Winder et al., 2012	[45]	Juvenile sheepshead minnows (*Cyprinodon variegates)*	0.3, 3, 30, and 300	84.41%(± 4.41) of nominal at t = 0 and 81.29% (± 3.39) of nominal at t = 56 h	56 hours	**300 µg/L:** ↓locomotor activity at 1, 25, 32, 49 and 56 hours post-dose
Wong et al., 2013	[39]	Male zebrafish (*Danio rerio)*	33	Not calculated	14 days	**All exposures**: ↓ time freezing, ↑ time spent in top half of NT, ↑ in IST and NPY, ↓ in URC,PRL, and GABA-transporter, *slc6a11* in whole brain. Genes associated with AA and lipid metabolism were ↑ and ↓, respectively. **No effect:** locomotor activity, 5-HT transporter in whole brain
Barry, 2013	[33]	Sub-adult Arabian killifish (*Aphanius dispar)*	0.03, 0.3 and 3	Not calculated	7 days	**3 µg/L:** ↓ Aggression **0.3 µg/L:** ↓ neighbour distance and swimming speed relative to neighbour
Weinberger and Klaper, 2014	[35]	Male and female fathead minnows (*Pimephales promelas)*	0.1, 1, 10, and 100	0.087 ± 0.017, 1.62 ± 0.42, 10.45 ± 2.83, and 94.85 ± 16.25	28 days	**≥1 µg/L:** ↑ nest cleaning and defending, ↓ mating behaviour in males. ↓ predatory avoidance **10 and 100 µg/L:** ↑ time to capture prey (following recovery, fish in 10 µg/L recovered)

					100 µg/L: ↑ aggression, isolation, and repetitive behaviours in males **No effect:** female mating behaviours, plasma T and estradiol levels
Forsatkar et al., 2014 [46]	Male Siamese fighting fish (*Betta splendens)*	0.54	Not calculated	6 days	**0.54 µg/L:** ↓ gill flaring, fin spreading and 90 degree turns following spawning
Mariotta-Casaluci et al., 2014 [43]	Male fathead minnows (*Pimephales promelas)*	0.1, 1.0, 8.0, 16, 32, 64	0.1, 1.2±0.3, 9.1±1.3, 17.4±2.2, 41.7±8.0 and 72.5±9.3	14 and 28 days	**64 µg/L for 14d:** ↑exploratory behaviour **32 and 64 µg/L for 28d:** ↑ exploratory behaviour **No effect**: speed
Sander de Abreu et al., 2014 [42]	Male and female zebrafish (*Danio rerio)*	1, 25, and 50	Not calculated	15 minutes	**All treatments:** Impaired whole body cortisol response to acute stressor **No effect:** whole body cortisol levels without stressor

↓: decrease
↑: increase
5-HT: 5-hydroxytryptamine
17,20p: 17a,20P-Dihydroxy-4-pregnen-3-o
AAs: amino acids
CRF: corticotropin-releasing factor
CYP: cytochrome
FK506: fujimycin
FSH: follicle stimulating hormone
FSHr: follicle stimulating hormone receptor
GABA: gamma-aminobutyric acid
GSI: gonadal somatic index
HSI: hepatosomatic index
IST: isotocin
LOEC: lowest observable effect concentration
LHr: luteinizing hormone receptor
NPY: neuropeptide Y
NT: novel tank
PG: prostaglandin
PGF2α: Prostaglandin F2alpha
PRL: prolactin
T: testosterone
URC: urocortin
VTG: vitellogenin

1.5. MicroRNAs in endocrine disruption

1.5.1. MicroRNA overview

MicroRNAs are evolutionarily conserved small non-coding RNA molecules that act as epigenetic regulators by altering mRNA translation and stability. Discovered only two decades ago by Lee et al. in the intron of a gene, the first miRNA molecule was originally thought to have no known function[47]. A decade later, miRNAs were regarded as a class of regulatory non-coding RNAs, part of a complex regulatory network and involved in the fine tuning of cellular processes[48].

Currently there are an estimated 2500 miRNA in the human genome on miRbase (http://www.mirbase.org/) and recently over 200 unique miRNAs were identified in Japenese medaka [49]. MicroRNAs regulate mRNA translation in the cell, with an estimated 30% of protein coding genes regulated by miRNAs[50]. MicroRNAs play a role in nearly all aspects of cellular functioning such as proliferation, migration and programmed cell-death [51–54]. MicroRNAs also have an important role in endocrine signalling, hormone metabolism and downstream signalling pathways[55]. The deregulation of miRNAs and their actions have been correlated with various disease states such as obesity, cancer and diabetes[56–59]. The analysis of miRNA action and function is a novel yet imperative consideration when investigating endocrine system disruption, hence its importance in this thesis as we consider metabolic disruption by an EDC, fluoxetine.

1.5.2. MicroRNA synthesis

MicroRNAs are approximately 20-22 nucleotides long and are products of multiple enzymatic reactions (see Fig. 1.4). Initially, RNA polymerase-II transcribes a large precursor molecule, primary-miRNA (pri-miRNA), from intergenic and intragenic regions on chromosomes in the nucleus [60]. The pri-miRNA precursor is a single long polycistronic primary

transcript and is adenylated following transcription [61]. Each of the miRNA sequences is 60-70 nucleotides long and eventually folds to form a stem-loop hairpin structure within the pri-miRNA. The stem loops are then recognized and undergo endonucleolytic cleavage by a multiprocessor complex containing an RNA-ase III, Drosha, and its co-factor, a double stranded RNA, DiGeorge critical region 8 [50]. The cleavage results in free small stem-looped miRNAs termed precursor-miRNAs (pre-miRNAs) [62]. Correctly spliced pre-miRNAs are then exported from the nucleus into the cytosol by the nuclear export factor exportin-5. Once in the cytoplasm, pre-miRNA undergoes a second enzymatic cleavage by the RNA-ase Dicer, which clips the stem loop that connects the 3' to the 5' strand of pre-miRNA. This cleavage results in a double stranded miRNA complex. The duplex miRNA will then bind to Argonaut ribonucleases in an RNA-induced silencing complex (pre-RISC), where the two strands finally separate into mature miRNA. The less stable miRNA will degrade, while the other will remain in the RISC and ultimately bind to and act on mRNA in the cytosol of a cell.

1.5.3. MicroRNA mechanism of action and subsequent biological action

Once properly synthesized, miRNAs act on mRNA targets in two main mechanisms: silencing mRNA translation or degrading mRNA molecules. In the first mechanism, a miRNA binds to the 3'-untranslated region (UTR) of its target mRNA to reduce stability and/or translation. Only a few base pairs, the seed region of 2-8 nucleotides, on the miRNA need to match to block the translational ribosome and cause RNA silencing[63]. In comparison, when a miRNA binds perfectly or near-perfectly with its target, an Argonaute2 endonuclease, Slicer, is activated and cleaves the mRNA molecule [55]. Though there has been a report of miRNAs increasing translation and up-regulating mRNAs, the majority of research is on the negative regulation of mRNA by miRNA[53]. See Fig. 1.5 for a summary of miRNA action.

The biological actions of mRNA targets are vast and encompass hormone responses, tissue development and disease[51,60,64]. MicroRNAs were recently discovered to regulate oocyte maturation and follicular development in humans, as well as steroid hormone receptor signalling pathways [64]. A recent review also outlined the role of miRNAs in the regulation of brain and cardiac development and the potential for therapeutic miRNA intervention [60]. MicroRNAs not only play a role in cellular action in healthy environments, but also can cause major upset when deregulated in pathological environments. MicroRNAs were determined to be key players in tumorigenesis and apoptosis associated with malignant cells and various forms of cancer [51]. These highlighted examples of miRNA action demonstrate their epigenetic regulator role and the importance of their consideration in hormone disruption studies.

1.5.4. MicroRNA and fluoxetine

Studies in mammals have found that fluoxetine alters miRNA levels in select tissues, demonstrating a link between miRNA and EDCs[65,66]. One study found that the effect of fluoxetine exposure on SERT translation is mediated by microRNA-16 (miR-16) in the raphe nucleus of mice [65]. Fluoxetine was found to inhibit canonical Wnt signalling, which normally negatively regulates pri/pre-miR-16 levels. By inhibiting Wnt signalling, fluoxetine increased miR-16 synthesis and subsequently decreased SERT mRNA translation. Conversely, fluoxetine can lower the levels of miR-16 in adjacent nuclei by promoting the translation of the neurotrophic factor S100b in the raphe nucleus. The S100b acts as a paracrine signal to inhibit miR-16 production in the locus coeruleus to promote serotonergic-specific functions. The effect of fluoxetine on miR-16 in the raphe nucleus also induces serotonergic neurons to secrete B-cell chronic lymphocytic lymphoma 2, which acts on the hippocampus in mice to reduce miR-16

production and causes an increase in hippocampal neurogenesis [66]. These findings show how fluoxetine can act on miRNA to cause physiological differences in the brain.

MicroRNA disruption caused by waterborne fluoxetine exposure has also been observed in zebrafish[6]. Craig et al. recently explored the effect of fluoxetine on hepatic miRNAs in zebrafish by use of a custom miRNA microarray to find potential key players in metabolic upset[6]. Following a 7-day waterborne exposure, six hepatic miRNA increased in fluoxetine exposed female zebrafish (*dre-miR-22b, dre-miR-140, dre-miR-210a, dre-mir-301, dre-miR-457b,* and *dre-let-7d).* Each miRNA was suspected to target dozens of mRNAs associated with metabolism as determined through target prediction and pathway analysis. In general, the miRNAs that were upregulated were predicted to be responsible for down-regulating pathways such as insulin signalling, cholesterol synthesis and triglyceride synthesis. In particular, two miRNAs, *dre-miR-140* and *dre-let-7d* were predicted to bind to mRNA subunits of the AMP-activated protein kinase (AMPK) enzyme, a master regulator of metabolism. An inverse relationship was found between the relative transcript abundance of the α1 and α2 mRNAs and *dre-miR-140* and *dre-let-7d*, suggesting that AMPK-related pathways may be compromised by fluoxetine exposure as a result of increased miRNA abundance in the liver. This study was the first to demonstrate miRNA as a potential mechanism in the metabolic upset caused by fluoxetine exposure in fish.

While miRNA sequences are conserved across species, the mRNA targets are not as well conserved. The 3'-UTR of mRNA is the binding target of miRNA and varies widely between species. As such, one miRNA in one species may alter a different pathway in another. The effect of fluoxetine on miRNA should therefore be investigated in a similar species to zebrafish and the mRNA targets should be evaluated to confirm if the fluoxetine-affected pathways are conserved.

This potential conservation of fluoxetine effect on miRNA and their targets will provide strong support for miRNA as an important endocrine disruption chemical target.

1.6. Metabolomics as a tool for endocrine disruption research

While evaluating single system endpoints such as reproductive behaviour and gonad physiology is valuable in EDC research, non-targeted techniques such as proteomics, genomics and transcriptomics are emerging as useful tools in discovering whole-system disturbances following EDC exposure. Rather than focussing on a small subset of potential changes, these analytical techniques effectively gain a more holistic understanding of EDC exposure, enabling researchers to determine novel mechanisms of toxicity [67] and evaluate EDC effects on multiple systems at once [68].

Metabolomics is the objective and comprehensive analysis of small molecules in a system (cell, tissue or organism) and has also recently begun to be used in EDC research. With the ability of metabolic fingerprinting to simultaneously evaluate metabolites belonging to several metabolic processes present in a complex sample, this tool considers the affected endocrine function at a molecular and phenotypic level. Metabolomics has already been successfully applied in medical endocrinology, where it is being used to predict diabetes onset [69], detect epithelial ovarian cancer [70] and as a proposed tool for individualised testosterone replacement therapy [71]. The metabolomic approach has also been widely applied in ecotoxicology studies and numerous review papers [72–77] have considered the benefits and pitfalls of using this -omics technique to evaluate toxicant exposure. Over the past few years, the advanced mass spectrometry based metabolomic approach has been increasingly and successfully applied in the investigation of EDCs in the environment, complimenting previous findings and providing direction for future studies [78–81].

The experimental design of the metabolomic fingerprinting approach to EDC exposure experiments is relatively consistent across studies. Organisms are exposed to varied levels of EDCs, tissues and/or biofluids are prepared for analysis and the small molecules within the prepared samples are separated, detected and statistically analysed using multivariate analysis to discover distinguishing markers (see fig 1.5). Metabolites that are significantly changed due to EDC exposure are then identified using online databases and pathway disturbances are revealed. Researchers may determine modes of action and detect new small molecules that may be useful as biomarkers in future studies and biomonitoring, all with an unbiased and exploratory methodology.

While this approach has potential to confirm previous EDC exposure findings and uncover novel information about molecular pathway disruptions, the technology used to separate and detect the metabolites in these experiments has yet to be standardized. The most commonly used analytical technologies used in EDC research are nuclear magnetic resonance (NMR)[82] and mass spectrometry (MS)[79]. The benefits and pitfalls of these approaches have been reviewed elsewhere [83,84]. Briefly, NMR is non-destructive, moderately reproducible, requires little sample preparation and is relatively precise in identifying metabolites from signal peaks [75]. However, NMR is less sensitive than MS and a larger sample volume is needed for signal separation and detection [77].

In comparison, MS based metabolomics analysis requires little sample volume and is capable of detecting hundreds to thousands of small molecules at once. Mass spectrometry is most often coupled with a separation technique such as liquid or gas chromatography (LC-MS or GC-MS) or capillary electrophoresis (CE-MS). Useful in EDC research, MS can detect low abundance molecules such as hormones and hormone metabolites. High Performance (HP) and

Ultra Performance (UP) LC-MS are now being used in metabolomic investigations and are able to separate thousands of metabolites in plants and animals. UPLC technology uses columns containing sub two micron sized-particles to improve resolution of compounds compared with LC and HPLC technologies[84]. This sensitive technique is also destructive, however, and requires extensive sample preparation which can introduce unwanted sample variation and can be relatively difficult in identifying distinguishing metabolites[75]. Due to the varied availability and use of these instruments in laboratories, there is yet to be an ideal technique for investigating EDC metabolomics. While no technique can detect the entire metabolome of an animal, combining these techniques such as NMR, LC-MS, and CE-MS can result in complimentary findings.

Following the separation of a solution, hundreds to thousands of individual small molecules can be detected per sample and appropriate statistical analysis is then applied to properly identify distinguishing metabolites between exposure groups. Statistical analysis is another area in metabolomics research that often differs between studies. Common tools are multivariate data analysis and pattern recognition such as principal component analysis (PCA) and orthogonal partial least square discriminate analysis (OPLS-DA), yet due to the differences in the use of these statistical tools, metabolites can be identified as discriminant in some studies yet overlooked in others.

The differences in statistical analysis, technologies, tissues and animal models can lead to unnecessary variability between studies, which is one of the major limitations of this approach in EDC research. Still, dozens of research groups continue to apply these hypothesis-producing techniques to EDC research in attempt to validate this approach and eventually come to a

consensus on optimal analysis techniques. Table 1.2 demonstrates the multitude of animals, EDCs, tissues, techniques and findings in metabolomic EDC research from 2008-2014.

The use of this exploratory approach has potential to aid in our research goal to determine the mechanisms of metabolic disruption caused by fluoxetine exposure by considering the end products, metabolites, in goldfish biofluids. This will be the first metabolomic evaluation of waterborne fluoxetine exposure in teleost fish to date. This research has potential to compliment previous fluoxetine exposure research, confirm our brain mRNA and hepatic miRNA results, and suggest potential biomarkers for future study in fluoxetine-induced metabolic disruption.

This research on waterborne fluoxetine exposure aims to uncover the mechanisms through which fluoxetine exerts its endocrine disruption effects on feeding and metabolism. By evaluating the feeding neuropeptides in the hypothalamus and telencephalon of goldfish brains following a short-term and environmentally relevant fluoxetine exposure, this investigation may clarify which feeding peptides are associated with suppression of appetite. Evaluating the hepatic miRNA in goldfish liver following short-term fluoxetine exposure will confirm a conservation of fluoxetine exposure effect on liver metabolism across fish species. Finally, examining the serum and bile metabolite profiles of goldfish following short-term fluoxetine exposure may put forth new biomarkers and potentially confirm previous research on fluoxetine-induced metabolism disruption. This whole systems approach may provide a holistic view on an important endocrine disrupting chemical.

Table 1.2: A list of disrupted metabolites caused by endocrine disrupting chemicals (EDCs) as detected by multiple metabolomic techniques from studies dating 2008-2014. Techniques used: proton nuclear magnetic resonance (H NMR), ultimate performance liquid chromatography (UPLC) time of flight (TOF) mass spectrometry (MS), gas chromatography (GC)/MS, High performance LC quadrupole TOF (HPLC-QTOF)/MS, capillary electrophoresis (CE)/MS, Electron spray ionization (ESI), reversed phase (RP) LC/MS, hydrophilic interaction chromatography (HILIC).

EDC	Authors	Ref	Organism	Tissue(s)	Technique(s)	Disrupted metabolite(s)
17α-Ethynyl-estradiol	Samuelsson et al. 2009	[82]	Rainbow trout (*Oncorhynchus mykiss*)	Blood	H NMR	Glucose, lactate, choline, cholesterol, lipoproteins, vitellogenin, alanine, phosphatidylcholine
	Ekman et al., 2008	[85]	Fathead minnow (*Pimephales promelas*)	Blood, liver, brain, pituitary, gonad	H NMR	Glyocogen, glucose, lactate (plasma), creatine, bile acids, glutamate, alanine (liver)
	Ekman et al., 2008b	[86]	Fathead minnow (*Pimephales promelas*)	Blood, liver	H NMR	Phosphatidylcholine, diglycerides, triglycerides, cholesterol
	Flores-Valverde et al., 2010	[81]	Roach (*Rutilus rutilus)*	Liver, gonad, bile, blood	UPLC-TOF/MS	Estrogens, androgens (bile and plasma), hydroxyprogesterone, androstenedione, 11-hydroxyandrostenedione, and 11-ketotestosterone (testes) cortisol, cortisone (testes, ovaries)
	Katsiadaki et al., 2010	[87]	Stickleback (*Gasterosteus aculeatus*)	Liver	H NMR	Glucose
	Teng et al., 2013	[88]	Zebrafish *(Danio rerio)*	Hepatocytes	H NMR	Alanine, glutamine, phosphocholine
	Leonard et al., 2014	[89]	Unionid mussel *(Lampsilis fasciola)*	Gill	GC/LC-MS, MS/MS	207 total: glycogen breakdown, amino acid metabolism, glucose, malate, essential fatty acids, glycerophospholipids, purine metabolism (see reference for full list)
Bisphenol A	Cho et al., 2008	[80]	Sprague-Dawley rat (*Rattus norvegicus*)	Urine	LC-MS	5-Hydroxymethyl-2'-deoxyuridine, 8-Hydroxy-2'-deoxyguanosine
	Chen et al., 2012	[79]	SD rat (*Rattus norvegicus*)	Urine, testis	HLPC-QTOF	Linoleic acid, arachidonic acid

	Cabaton et al. , 2013	[78]	CD-1 mouse *(Mus muscaris)*	Body, blood, brain, liver	H NMR	Aspartic acid, cholines, creatine, GABA, glucose, glutamate, glutamine, glutathione, glycine, glycogen, lactate, lipids, lipoproteins, taurine, valine, leucine, isoleucine, lycine
	Zeng et al., 2013	[90]	SD rat (*Rattus norvegicus*)	Urine	CE-TOF/MS	Various AAs, amino ketones, polyamines, nucleosides, organic acids, carbohydrates, pterins, polyphenols, sugar phosphates, noradrenaline, GABA, glutamate, histadine
	Chen et al., 2014	[91]	SD rat (*Rattus norvegicus*)	Urine	CE-ESI/TOFMS	Biotin, riboflavin, methylated products, choline metabolites
	Ji et al., 2014	[92]	Mussel (*Mytilus galloprovincialis*)	Gonad	H NMR	Valine, leucine, isoleucine, hypotaurine, choline, betaine, threonine, glycogen, homarine, proline, alanine, histidine
Methoxyclor	Kim et al., 2009	[93]	Rat (*Rattus norvegicus*)	Urine	H NMR	Acetate, alanine, 2-oxoglutarate, allantoin, citrate, formate, benzoate, lactate, succinate, taurine, glycine, phenyl acetate
Atrazine	Ralston-Hooper et al., 2011	[94]	Scud (*Hyalella azteca*)	Whole body	GCxGC and LC/TOF-MS,	Eicosanoids, fatty acid metabolites
PCBs & TCDD	Lu et al., 2010	[95]	Rat (*Rattus norvegicus*)	Urine	H NMR	lactate, glucose, taurine, creatine, and 2-hydroxy-isovaleric acid, 2-oxoglutarate, citrate, succinate, hippurate, trimethylamine-N-oxide
Fenitrothion	Southam et al., 2011	[96]	Roach (*Rutilus rutilus)*	Brain, testis, liver, blood	H NMR, LC/MS, DIMS	Creatine, phosphocreatine, isoleucine, valine, phosphogen and phenylalanine metabolites (liver), 11-ketotestosterone (plasma) and cortisone (testes)
Phthalates & PCB	Zhang et al., 2012	[97]	Kumming mice *(Mus muscaris)*	Blood	LC/MS: RP & HILIC	Phospholipids, tryptophan, phenylalanine
PBDE (BDE 47)	Ji et al., 2013	[98]	Mussel (*Mytilus gollaprovincialis)*	Gill, gonad	H NMR	Glutamate, threonine, lysine, glucose, histidine, betaine, ATP, hypotaurine

PBDE: Polybrominated diphenyl ethers

PCB: Polychlorinated biphenyls

TCDD: 2,3,7,8-tetrachlorodibenzo- p-dioxin

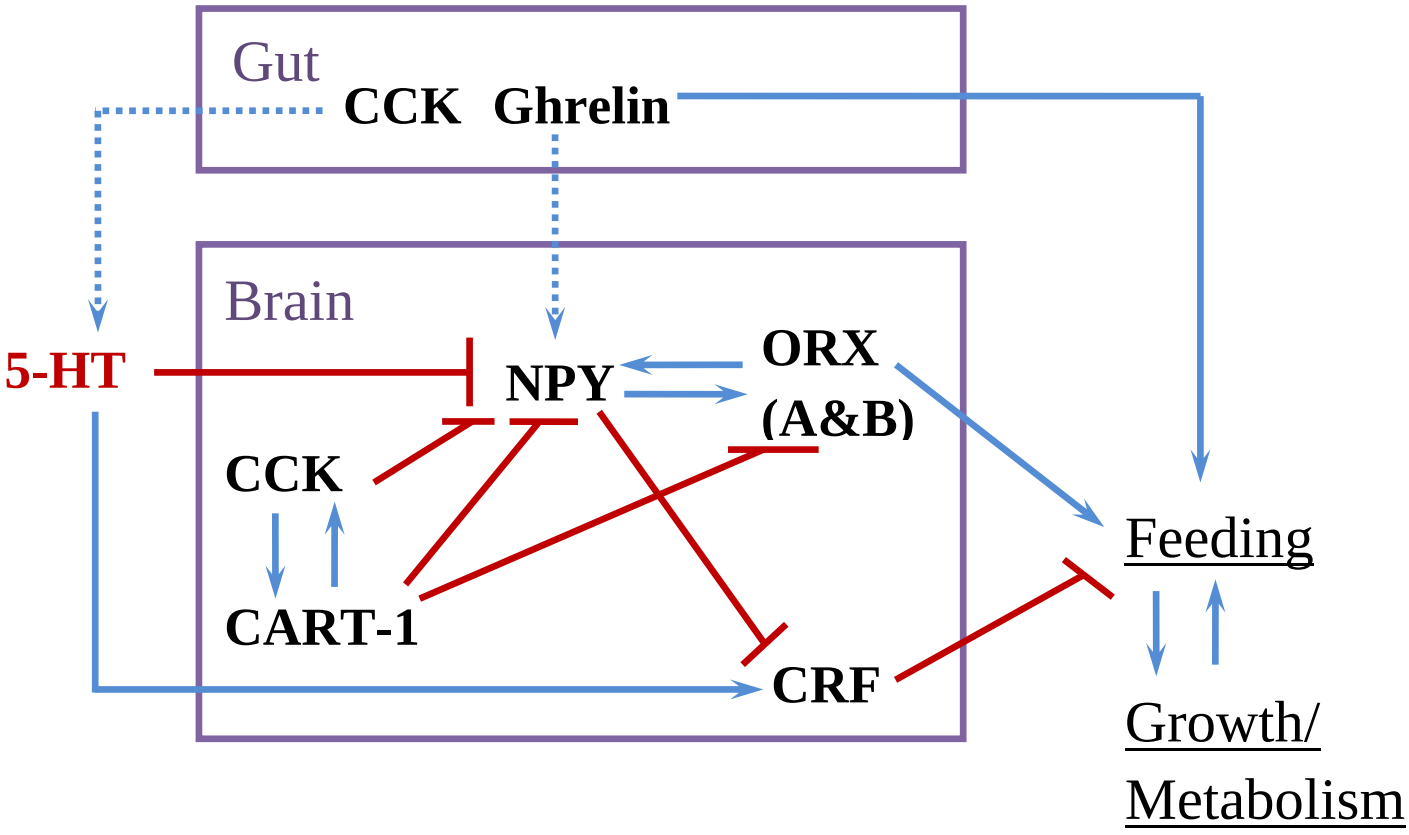

Figure 1.1. The influence of serotonin (5-HT) on the neuroendocrine feeding pathway in goldfish with proposed pathways in dotted lines, inhibitory pathways in red, and inducing pathways in blue. CART-1: cocaine and amphetamine- regulated transcript, CCK: cholecystokinin, CRF: corticotropin-releasing factor, NPY: neuropeptide-Y, ORX: orexin.

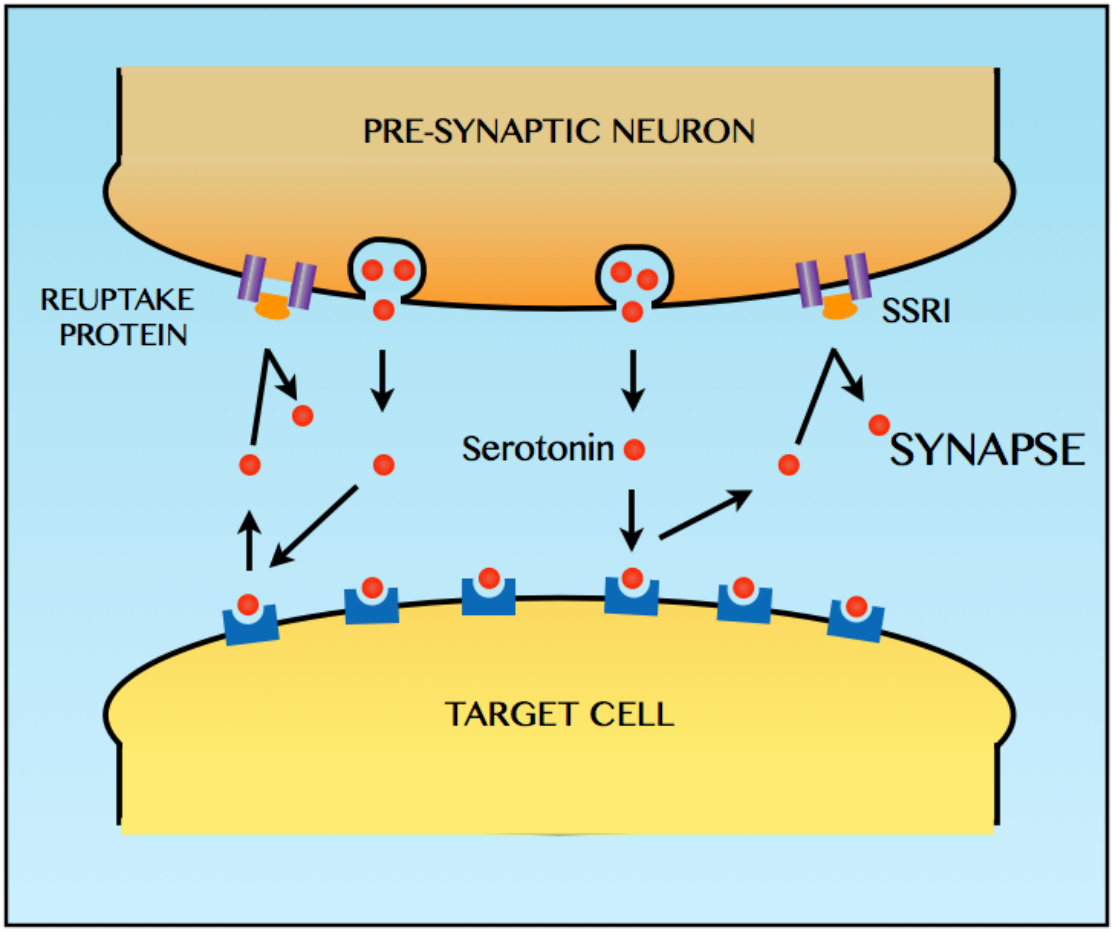

Figure 1.2. Mechanism of action of fluoxetine, a selective serotonin re-uptake inhibitor (SSRI). Fluoxetine binds to the serotonin reuptake transporter (SERT) on the pre-synaptic neuron, allowing for the neurotransmitter serotonin to continue binding to target cell receptors.

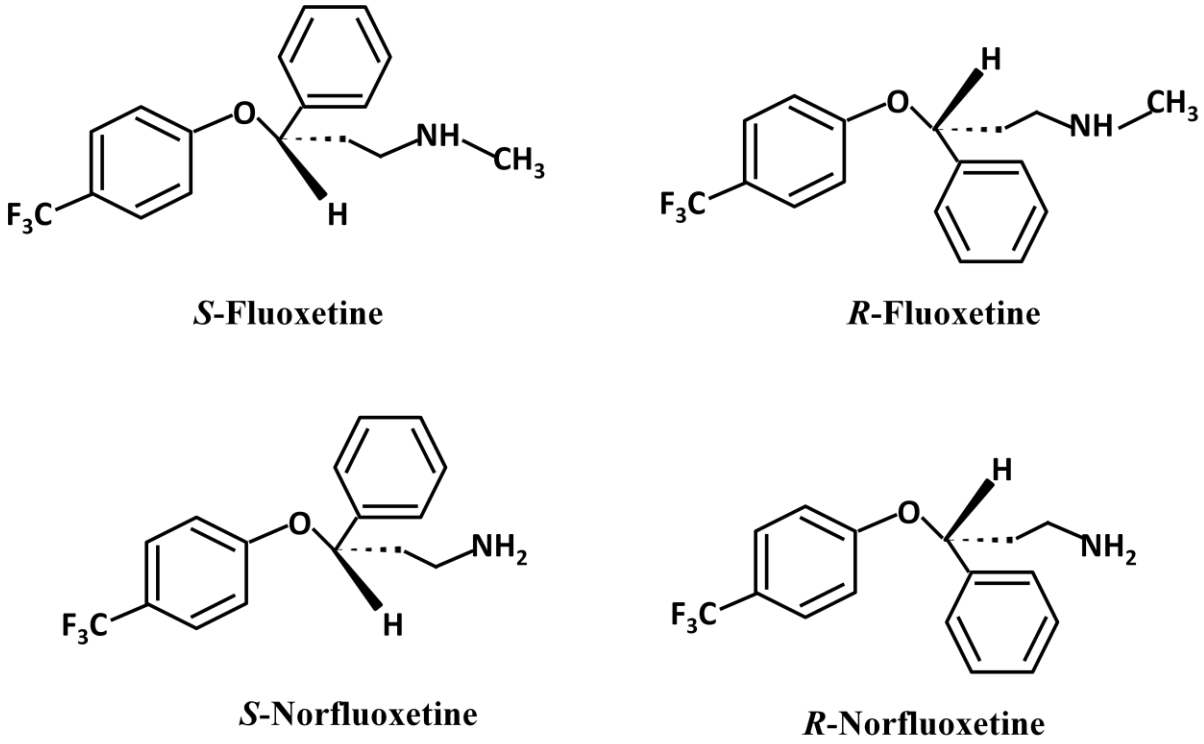

Figure 1.3. S- and R- enatiomers of fluoxetine and its demethylated metabolite, norfluoxetine.

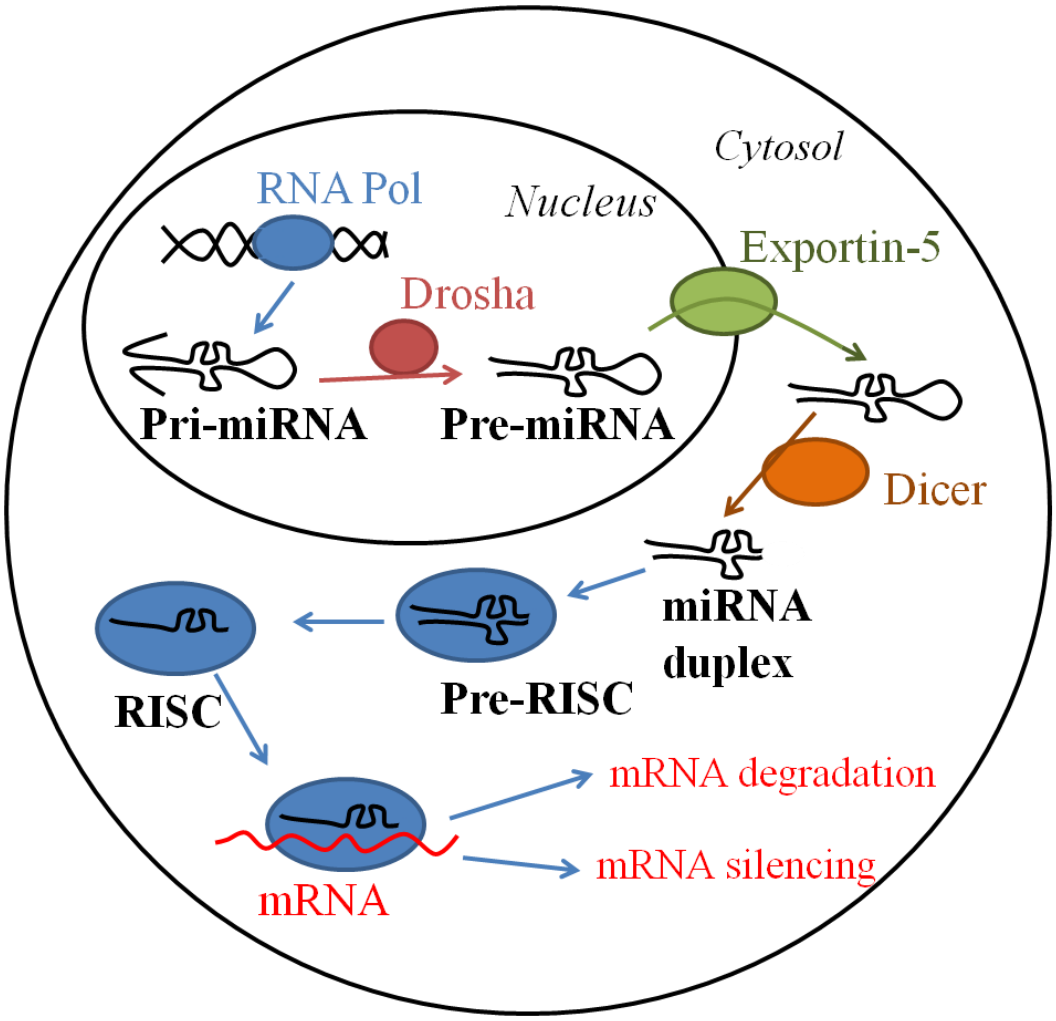

Figure 1.4. MicroRNA biosynthesis pathway. Primary microRNA (pri-miRNA) are transcribed by RNA Polymerase II in the nucleus and cut into pre-miRNA by Drosha. Exportin-5 transports pre-miRNA into the cytoplasm, where Dicer further splices the molecules into miRNA duplexes. The miRNA duplex forms a pre-RNA inducing silencing complex (RISC) before the less stable miRNA strand is degraded. The resulting RISC complex binds to the seeding sequence of target mRNA to result in mRNA degradation or silencing.

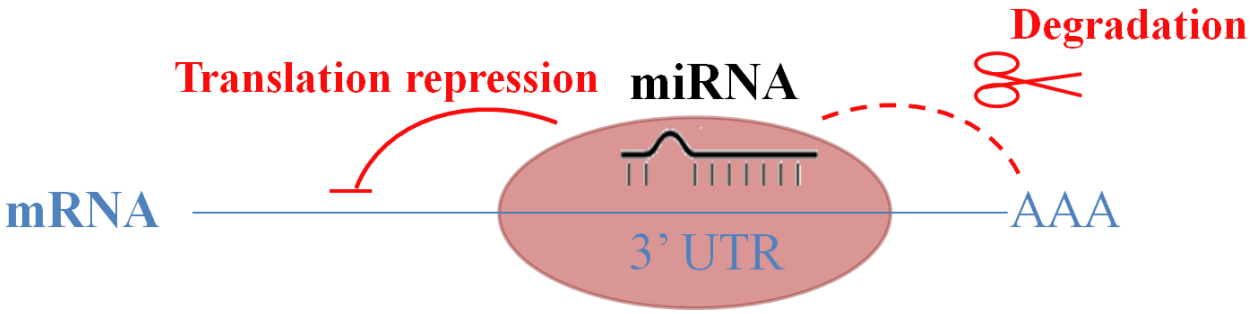

Figure 1.5. The action of microRNA (miRNA) on its messenger RNA (mRNA) target. UTR: untranslated region.

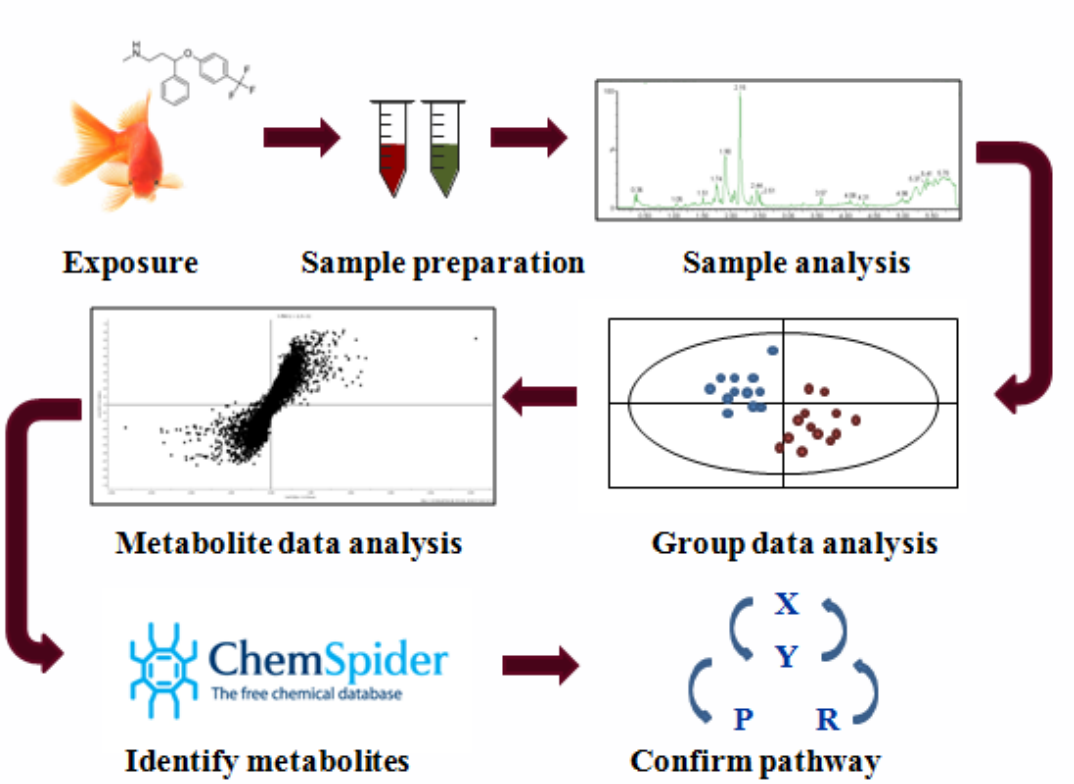

Figure 1.6. The generalized methodology for applying a metabolomic approach to endocrine disruption caused by environmental contaminants. Organisms are exposed to varied levels of EDCs, tissues are prepared for analysis and the small molecules within the prepared samples are separated, detected and statistically analysed using multivariate analysis to discover distinguishing markers. Metabolites that are significantly changed due to EDC exposure are then identified using online databases and pathway disturbances are revealed.

CHAPTER 2:

Short-term waterborne fluoxetine exposure increases anorexigenic neuropeptide mRNA and hepatic miRNA associated with metabolic upset in *Carrasius auratus*

2.1. Introduction

Antidepressants are found in the aquatic environment at concentrations capable of disrupting endocrine and neural systems of exposed animals. All of the antidepressants combined in watershed downstream of a waste water treatment plant can reach as high as 1 μg/L in some great lake areas of Canada[99]. Fluoxetine, the active ingredient in Prozac®, has been found in North American waters up to 0.54 μg/L and has been the subject of ecotoxicology studies for nearly a decade due to its endocrine disruption effects[23]. Reproduction, anxiety, aggression and stress are all fluoxetine-affected systems that have been previously outlined in the literature[36,41,42,100]. The specific effects of fluoxetine on feeding and metabolism, however, are relatively vague and unknown, with only five studies in the past ten years addressing this area[2,5,6,31,39]. In general, waterborne fluoxetine exposure decreases weight gain and feeding motivation in aquatic organisms and down-regulates metabolic pathways in the liver such as gluconeogenesis[5].

Fluoxetine is an SSRI that reduces feeding motivation in fish through its action on 5-HT signalling. By determining the expression levels of several feeding neuropeptides in the hypothalamus and the telencephalon following short-term fluoxetine exposure, this study examines which areas of the feeding cascade are initially affected by fluoxetine. We also consider the effects of fluoxetine on liver function by evaluating hepatic miRNA levels to confirm that these epigenetic regulators play a role in fluoxetine-induced metabolism disruption at the level of the liver. This investigation aims to uncover the intermediate steps in the

downstream disruption of feeding and metabolic activity caused by waterborne fluoxetine exposure in goldfish.

The mRNA abundance of seven neuropeptides were investigated based on our understanding of the inhibitory actions of 5-HT on the feeding circuits in the goldfish brain (see section 1.3). Feeding motivation is regulated by orexigenic (induces appetite) and anorexigenic (inhibits appetite) neuropeptides. The three orexigenic peptides considered in this study were neuropeptide Y (NPY), orexin-A and secretogranin-IIa (SgIIa). Neuropeptide Y is the most potent orexigenic peptide in fish and its expression undergoes periprandial variations in the brain[15]. Neuropeptide Y mRNA levels in the telencephalon–preoptic area and hypothalamus of goldfish increase shortly before feeding followed by a decrease shortly after[101]. Fluoxetine exposure has been shown to decrease NPY mRNA at environmentally relevant levels in goldfish telencephalon and zebrafish whole brain, though this effect is reversed at higher doses [5,39]. Orexin-A mRNA abundance shows similar periprandial trends before and after feeding[102]. Injections of the orexin-A and -B induce feeding and increase locomotor activity in goldfish [103]. No studies have investigated the effects of fluoxetine exposure on the orexins.

Secretogranin IIa (SgIIa), the precursor of secretoneurin A (SNa) was also considered. Secretoneurin is a moderately conserved peptide across vertebrates that has recently been proposed to play a role in feeding behaviour[104]. Injections of SNa into the third ventricle in goldfish brain increases feeding and locomotor behaviour. In addition, SNa injections increased NPY mRNA levels in the hypothalamus demonstrating a co-activation of the feeding system in goldfish. Secretogranin-IIa was selected for analysis due its moderate increase in mRNA abundance in hypothalamus and telencephalon following short fasting experiments using female goldfish and as a potential mechanism for fluoxetine-induced decreases in food intake[104]. These

orexiogenic neuropeptides are hypothesized to decrease in brain tissues following fluoxetine exposure because 5-HT and fluoxetine are appetite suppressors.

Four anorexigenic neuropeptides were also investigated: corticotropin-releasing factor (CRF), cholecystokinin (CCK), cocaine- and amphetamine-regulated transcript-1 (CART-1) and isotocin. Corticotropin-releasing factor is a well known neuropeptide in the hypothalamic pituitary interrenal (HPI) axis, where it promotes adrenocorticotropin hormone (ACTH) release from the pituitary, leading to the eventual release of cortisol from the interrenal glands. An extra-HPI function of the CRF system is in the regulation of food intake and energy balance[105]. Corticotropin-releasing factor is known to inhibit food intake and long-term fluoxetine exposure has been found to down-regulated CRF mRNA in female goldfish hypothalamus[5], making it an important candidate in this short-term fluoxetine exposure investigation.

Cholecystokinin is another anorexigenic peptide in teleost fish and acts on CCK-A receptors in gut and CCK-B receptors in brain to inhibit feeding response[15]. In goldfish, CCK mRNA is widely expressed in the brain, with the highest levels found in the hypothalamus. Injections of CCK inhibited goldfish feeding[106], while oral feeding of CCK antagonists induced food intake in rainbow trout[107]. No effects of fluoxetine on CCK have been examined as of yet.

Another peptide of interest is CART-1 as it causes a decrease in feeding behaviour when injected into goldfish brain, increases in mRNA abundance following feeding and decreases following food deprivation[102]. The anorexigenic properties of CART-1 may be through its inhibitory effect on the orexigenic properties of NPY and orexin-A. When co-injected with NPY and orexin-A, CART-1 reduces their orexigenic effects in goldfish[102]. Injections of the orexigenic SNa into the goldfish brain also caused decreases of CART-1 mRNA expression[104].

Injections of fluoxetine in female goldfish cause increases of CART-1 mRNA in the telencephalon[108]. No waterborne fluoxetine studies have considered CART-1 as of yet.

Lastly, isotocin was selected as an anorexigenic neuropeptide to evaluate as previous research suggested isotocin plays a role in feeding regulation. Isotocin is the fish homolog to mammalian oxytocin and is primarily known for its role in reproduction. Isotocin may be a key player in feeding motivation due to its interactions with 5-HT. Mennigen et al. (2008) found that a two-day fasting period decreased the amount of isotocin mRNA in the hypothalamus[37]. Contrary to this, however, injections of the assumed anorexigenic fluoxetine caused isotocin mRNA levels to decrease in the hypothalamus and telencephalon, as did high doses of fluoxetine during a 28-day waterborne exposure[3,37]. As such, the role of isotocin in fluoxetine-induced anorexia is still unclear and warrants further investigation. These anorexigenic neuropeptides are hypothesized to decrease following fluoxetine exposures in goldfish.

In addition to the effects of fluoxetine on feeding peptide levels in the brain, this investigation also considers the effects of fluoxetine on liver function. Long-term waterborne fluoxetine exposure was previously found to up-regulate hexokinase and glucokinase activity in goldfish compared to fasted groups[5]. The specific target of fluoxetine, *slc6a4a* (SERT), is found in goldfish liver[3] and fluoxetine is known to bioconcentrate in the livers of goldfish, killifish, rainbow trout, Japanese medaka and zebrafish[4,7,29–31]. Fluoxetine is therefore likely to act directly or indirectly at the liver by upsetting 5-HT signalling to disrupt liver metabolism.

To dissect the mechanisms behind the effect of fluoxetine on liver metabolism, we focus on an intermediate molecule in endocrine and metabolic pathways, miRNA. These small non-coding molecules act as epigenetic regulators and have only recently been discovered to be part of hormone regulation such as thyroid hormone, luteinizing hormone and estrogen[50,109,110].

MicroRNAs down-regulate mRNAs and their associated pathways by either blocking translation or causing degradation of their mRNA targets. Chemicals alter miRNA levels by up or down-regulating their synthesis, thus increasing or decreasing their abundance and action on their mRNA targets. Recently, the effects of fluoxetine on hepatic miRNAs and their mRNA targets were considered by Craig et al.[6]. Following a short-term and environmentally relevant waterborne fluoxetine exposure, a microarray identified six hepatic miRNAs that were significantly increased in female zebrafish liver (*dre-miR-22b, dre-miR-140, dre-miR-210a, dre-mir-301, dre-miR-457b* and *dre-let-7d*). The predicted mRNA targets in zebrafish coded for important peptides in metabolic pathways, such as cholesterol biosynthesis, insulin signalling and triacylglyceride synthesis. As miRNA are highly conserved across species, the sequences found in zebrafish were likely to match those found in goldfish. To consider if the effect of fluoxetine on these miRNA was conserved, the six hepatic miRNAs were quantified in our goldfish following the short-term fluoxetine exposure.

In this study, male and female goldfish were placed into three separate exposure groups (0, 0.5, and 1 μg/L fluoxetine) for seven days. Following the exposure, hypothalamus and telencephalon tissues were evaluated for NPY, orexin-A, SgIIa, CRF, CCK, CART-1, and isotocin mRNA abundance. The results will put forward which sections of the feeding circuit in the goldfish brain are regulated during the early stages of fluoxetine exposure. Livers were also examined for the abundance of six miRNAs: *dre-miR-22b, dre-miR-140, dre-miR-210a, dre-mir-301, dre-miR-457b* and *dre-let-7d* and expected mRNA targets are currently being determined through target prediction. A separate, male-only 14-day fluoxetine exposure was also conducted and livers were evaluated for hepatic miRNA abundance. These findings will confirm whether there is a conserved effect of fluoxetine on the abundance of these hepatic miRNAs and help to

strengthen the proposal that miRNA are a potential route through which fluoxetine alters metabolic activity[6].

2.2. Materials and methods

2.2.1. Experimental design

Common adult goldfish, *Carassius auratus*, were purchased from Mt. Parnell Fisheries and supplied by AQUAlity Tropical Fish Wholesale (Mississauga, ON, Canada) and acclimated for at least three weeks prior to handling at the University of Ottawa Aquatic Care Facility. Fish were kept in 70 L tanks in groups (9-12 fish per tank) in 18 °C single-use flow-through (300 mL/min/tank) dechloraminated City of Ottawa water under a natural simulated seasonal diurnal photoperiod. Goldfish daily diet was Martin Proficient Classic Floating Trout Grower 3 mm pellets (Martin Mills Inc., Travistock, ON, Canada), which contain 44% crude protein, 0.45% sodium, 10% crude fat, 3.5% crude fibre, 0.9% calcium, 0.97% phosphorous, 6800 IU/kg Vitamin A, 2100 IU/kg Vitamin D3 and 320 IU/kg Vitamin E. Three days before the exposure, selected goldfish were weighed and randomly assigned to aerated static water tanks with 80% water renewals every two days. Feeding was regular (3PM daily, 36 pellets per tank of 12 fish) starting three days before the exposure and throughout, with no change in feeding habits in any tank. At the end of the exposure, fish were anesthetised in water containing 0.1 g/L 3-aminobenzoic acid ethylester (MS-222 Aqua Life, Syndel Laboratories Ltd., Vancouver, BC, Canada) before any dissection procedures. Care was taken to standardize all handling protocols. All procedures were approved by the University of Ottawa Protocol Review Committee and followed standard Canadian Council on Animal Care guidelines.

2.2.1.1. 7-day exposure

In August 2013, a 7-day exposure was performed with both male and female goldfish (n=72). Fish were weighed and randomly placed in one of three exposure groups: control (0 μg/L fluoxetine), low (0.5 μg/L fluoxetine) and high (1 μg/L fluoxetine). The low concentration represents the highest environmental concentration in North American waters as reported by Brooks et al.[23] and the high concentration represents the maximum total concentration of all antidepressants in the environment, as reported by Metcalfe et al. (2003) in Canadian wastewater[99]. Before the exposure began, there was no significant difference between group whole body mass average (one-way ANOVA, $p>0.05$, data not shown). Following three days of acclimation, fluoxetine hydrochloride (Drogueria Saporiti, Buenos Aires, Argentina) was weighed and dissolved in water to a stock concentration of 7 μg/mL. Using a pipette, 5 mL and 10 mL of the stock solution were added to the 70 L low and high exposure tanks for final nominal tank concentrations of 0.5 and 1 μg/L, respectively. Fluoxetine hydrochloride is water soluble with a water solubility of 4mg/mL, so the control was aquarium water without any additions. Paterson and Metcalfe[30] and Gaworecki and Klaine[2] estimated the half-life of fluoxetine in populated fish tank water at approximately 3 days; therefore, every two days, 80% of the tank water was drained and new water was added followed by a re-addition of a new fluoxetine solution.

Following the exposure, fish were anesthetised in water containing 0.1 g/L 3-aminobenzoic acid ethylester (MS-222) and weighed. Blood (100-600 μL) was collected by caudal puncture with a 26-gauge needle and was kept on ice to allow for clotting. Blood samples were then centrifuged for 15 min at 3000 g (8200 rpm) and 4 °C and serum was removed and stored at −20 °C for metabolomic analysis (Chapter 3). Fish were sacrificed by spinal

transsection and the hypothalamus, telencephalon and liver were removed and immediately frozen on dry ice before being stored at −80 °C until RNA isolation. Gonad tissue was also removed and weighed to determine gonadosomatic index (GSI). Bile (5-100 µL) was removed by syringe (28-gauge) and placed on ice, centrifuged with the serum parameters and stored at −20 °C for metabolomic analysis (Chapter 3). Tools used were dipped in 3% H_2O_2 and DEPC water (x2) to ensure the destruction of RNAses before each use.

2.2.1.2.14-day exposure

The 14-day exposure used males only (n=27) due to the lack of available females in the aquatic facilities. The exposure was conducted in May 2013, with lights synchronized to Ottawa, ON, Canada day length. Males were identified by the presence of breeding tubercles on pectoral fins, weighed and randomly placed in one of three exposure tanks: control (0 µg/L fluoxetine), low (0.5 µg/L fluoxetine) and high (1 µg/L fluoxetine). The steps taken in preparing the fish for the exposure were the same as the 7-day experiment. Before the exposure, average body mass was not significantly different across treatment groups ($p>0.05$, data not shown). The same static renewal of water and fluoxetine treatment was performed and the same dissection procedures were followed as the 7-day experiment. At the time, no hypothalamus and telencephalon tissue samples were taken.

2.2.2. Total mRNA extraction, cDNA synthesis and real-time RT-PCR

Total RNA was extracted from hypothalamus and telencephalon tissues of male (n=6-8) and female (n=8) goldfish from the 7-day exposure using RNeasy Plus Micro kit (QIAGEN, Mississauga, ON, Canada) following the manufacturer's protocol. Following extraction, concentration and quality of all samples were assessed with NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and selected RNA samples were run on the 2100

Bioanalyzer to confirm RNA integrity. All selected samples had RNA integrity numbers (RIN) above 7. Complimentary DNA (cDNA) were prepared from 1 µg RNA using the cDNA synthesis kit (Thermo Scientific, Ottawa, ON) and were synthesized using the Mastercycler ® gradient Thermal Cycler (Eppendorf, Westbury, NY, USA) (10 minutes at 25 °C, 30 minutes at 50 °C, 5 minutes at 80 °C). 20 µL reactions were then diluted into 180 µL nuclease-free water and frozen at -20°C until needed for real-time quantitative polymerase chain reaction (RT-qPCR).

Real-time-qPCR assays based on SYBR green detection were used to confirm expression of seven genes (*ß-actin, crf, orexin, sgIIa, cart1, cck, npy, ist).* Sequences for genes of interest were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/genbank) and if previous goldfish studies had not reported primers, new ones were designed using Primer 3 (bioinfo.ut.ee/primer3-0.4.0) and synthesized by Invitrogen (Table 2.2). The Maxima SYBR green qPCR Master Mix (Thermo Scientific, Ottawa, ON, Canada) and Mx3000 Quantitative PCR System (Stratagene, La Jolla, CA) were used to amplify and detect the selected transcripts. The thermal cycling parameters were: 1 cycle Taq activation at 95°C for 15 min, 40 cycles of 95°C for 15 seconds, followed by optimized annealing temperature (60-63°C) for 5 seconds, 72°C for 30 seconds, and a detection step at 80°C for 8 seconds. Data were analyzed using the MxPro software package (Agilent). Relative mRNA abundances were normalized by using NORMA-GENE algorithm[111] and were compared to the levels of the reference gene, beta-actin. All samples were assayed in duplicate, with an n of 6-8 for each group.

Table 2.1. Messenger RNA primers, their sequences and GenBank accession numbers (#) used for qPCR quantification.

Gene	Forward primer sequence (5'3')	Reverse primer sequence (3'5')	GenBank accession #
b-a	CTGGGATGATATGGAGAAGA	CCAGTAGTAC GACCTGAAGC	AB039726.2
cart-1	CCATGGAGAGCTCCAAACTC	TCTTGACCCTTTCCTGATGG	AF288810
cck8	AACGCTGGAATCTGTGTGTG	GGGGCTCTTCATCATCCTCT	U70865.1
crf	TTCGGGAAGTAACCATGAGC	TGATGACAGTTTTGCGCTTC	AF098629
ist	ATCTTGGCTACTGGCAGCTT	GTATCTGCTGTGGTGAAGGT	U70865.1
npy	TTCGTCTGCTTGGGAACTCT	CTGGGGATGGGACTCTGTTT	M87297
orexin-a	ACTGCACAGCCAAGAGAGTTCA	GTTATTAAAGCGGCCGATATGC	DQ923590.1
sg-IIa	CCTCAGCCAGAGAACTCCAC	ATGCCTCTATCCATCCGAGA	AF046002.1

b-a: beta-actin

2.2.3. Total miRNA extraction, cDNA synthesis and real-time RT-PCR

Total miRNA was extracted from liver tissues of males (n=5) from 14-day exposure using Absolutely RNA miRNA kit (Agilent). Due to the difficulty in achieving uncontaminated samples using this kit as determined by low 260/280 values, the miRNeasy Mini kit (QIAGEN, Mississauga, ON, Canada) was used for liver tissues from the males and females (n=7, n=8 respectively) from the 7-day exposure. Following miRNA isolation, concentration and quality of all samples were assessed with NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and selected RNA samples were run on the 2100 Bioanalyzer to confirm RNA integrity. All selected samples had RIN values above 7. Complimentary DNA synthesis was completed using the miScript II RT Kit (QIAGEN, Mississauga, ON). RNA samples were mixed with 5x Hi Spec, 10x Nuclear Mix, RT Mix and water to amount to 1 μg of RNA, and were gently mixed, briefly centrifuged and placed into the Bio-Rad PCR Machine for 60 minutes at 37°C and 5 minutes at 95°C. Samples were frozen at -20°C until needed for RT-PCR.

Real-time RT-PCR assays based on SYBR green detection were used to validate relative expression of six miRNAs (*dre-miR-22b, dre-miR-140-5p, dre-miR-210-5p, dre-miR-301a, dre-miR-457b,* and *dre-miR-let-7d*). Genes of interest and specific primers were selected from Craig

et al[6] and primers used for RT-PCR were synthesized by QIAGEN (see table 2.2). The "dre" nomenclature was kept as these genes have not yet been sequenced in goldfish. Real-time qPCR was performed using the Rotor-Gene Q real-time PCR thermocycler (QIAGEN) and Rotor-Gene SYBR green PCR kit (QIAGEN). Each reaction contained 5 µl Rotor-Gene SYBR green PCR master mix (QIAGEN), 1 µl of both forward and reverse gene specific primers for a final concentration of 1 µM, 2 µl RNase/DNase-free purified water and 1µl cDNA. Cycling conditions were: 5 minutes initial denaturation at 95°C, 40 cycles of 95 °C for 5 seconds, and 60 °C for 10 seconds. Additionally, for validation that only one product was amplified, a melt curve analysis was used at the end of each run. Each sample (n = 5-8) was run in duplicate. The relative standard curve method was used to calculate relative mRNA abundance between samples, which were normalized by using NORMA-GENE algorithm[111].

Table 2.2. MicroRNA, their sequences and their miRBase number (#) used for qPCR quantification.

Gene	Sequence	miRBase #
dre-miR-22b	AAGCTGCCAGTTGAAGAGCTGT	MIMAT0001789
dre-miR-140-5p	CAGTGGTTTTACCCTATGGTAG	MIMAT0001836
dre-miR-210-5p	AGCCACTGACTAACGCACATTG	MIMAT0003392
dre-miR-301a	CAGTGCAATAGTATTGTCAAAG	MIMAT0001870
dre-miR-457b	AAGCAGCACATAAATACTGGAG	MIMAT0001884
dre-miR-let-7d	TGAGGTAGTTGGTTGTATGGTT	MIMAT0001762

2.2.3. Statistical analysis

Statistical analysis was performed using SigmaPlot software (Systat Software, San Jose, CA). Data were tested for normality using the Shapiro-Wilk normality test. Non-normal data were transformed using either log10 or square root as required for parametric statistical analysis. All 7-day male and female body mass and GSI data were compared using two-way ANOVA with Bonferonni post-hoc to analyze sex and exposure differences. The remaining data were compared using one-way ANOVA with Tukey's post-hoc for each individual sex and tissues. Results were considered significant when $p<0.05$.

2.3 Results

All normally-distributed data were presented as means + SEM for each sample group. If samples groups were not normally distributed, individual sample points were plotted and the median was presented.

2.3.1. Goldfish mass and GSI following fluoxetine exposures

2.3.1.1. 7-day exposure

No overall body mass difference between exposure groups was observed following the 7-day exposure ($p>0.05$), with an average body mass for males and females of 17.88 ± 0.37 g. Following the 7-day treatment, females weighed significantly more than males in the control group but not in either exposure group, demonstrating a significant interaction between dose and sex ($p<0.05$) (Fig. 2.1A). There was no significant difference across exposure group for GSI for either sex, which averaged 1.31 ± 0.15% ($p>0.05$). There was a trend ($p=0.054$) for males to have lower GSI in the fluoxetine exposure groups compared to control. Females had significantly higher GSI than males in low and high exposure groups but not in control ($p<0.05$) (Fig. 2.1B).

2.3.1.2. 14-day exposure

No significant differences in male average body mass between exposure groups was observed following the 14-day exposure ($p>0.05$) (Fig. 2.2A). Gonadosomatic index was significantly lower in the low fluoxetine exposure group than the control and high exposure groups ($p<0.05$) (Fig. 2.2B).

2.3.2. Neuropeptide levels in male and female goldfish hypothalamus and telencephalon following 7-day fluoxetine exposure

Male and female data were analyzed separately. None of the three orexigenic neuropeptides investigated showed any significant changes in either male or female

hypothalamus and telencephalon tissues ($p>0.05$). Neuropeptide Y mRNA showed a trend to decrease in low exposure groups in male hypothalamus, though this effect on NPY mRNA abundance was no longer present in the high exposure groups (Fig. 2.3A). Neuropeptide Y mRNA showed no significant trends in female hypothalamus (Fig. 2.3B), nor in the telencephalon of either sex (Fig. 2.3C, D). Orexin-A mRNA and SgIIa mRNA showed no major trends in either tissue or sex (Fig. 2.4 and Fig. 2.5).

Two out of the four anorexigenic neuropeptides showed significant differences between exposure groups. While there was no significant effect in the males in either tissue (Fig. 2.6A,C), CRF mRNA levels significantly increased 2-fold in the high exposure group in female hypothalamus (Fig. 2.6B) and telencephalon (Fig. 2.6D). Isotocin mRNA increased in male hypothalamus by 3-fold, though the difference did not reach statistical significance ($p>0.05$). Isotocin mRNA did not differ in female nor male telencephalon (Fig. 2.7A-C), however isotocin mRNA abundance was 2-fold higher in the high exposure female telencephalon compared to the low exposure female telencephalon ($p<0.05$) (Fig. 2.7D). The other anorexigenic neuropeptides CCK and CART-1 showed no significant differences in mRNA abundance in either sex or tissue (Fig. 2.8 and Fig. 2.9).

2.3.3. Hepatic miRNA levels in male and female goldfish liver following fluoxetine exposures

Male and female miRNA abundances in the liver were compared separately. Following the 7-day fluoxetine exposure, female hepatic miRNA abundance increased significantly (4-6 fold) for *dre-miR-22b, dre-miR-140, dre-miR-210, dre-miR-301a* and *dre-miR-457b* (Fig. 2.10). There were further significant differences between low and high exposure groups for two (*dre-miR-301a* and *dre-miR-457b*) of the six hepatic miRNA studied. No significant changes were observed in *dre-let-7d* in females following the fluoxetine exposure.

In male goldfish, the 7-day fluoxetine exposure significantly increased one out of the six miRNA, *dre-miR-22b* by 4-fold, while the other five showed trends of increasing without statistical significance (Fig. 2.11). Following the 14-day fluoxetine exposure, male hepatic miRNA abundance increased significantly by 2-fold for *dre-let-7d, dre-miR-210, dre-miR-301a,* and *dre-miR-457b* (Fig. 2.12).

2.4 Discussion

2.4.1 Short-term fluoxetine exposure did not affect goldfish body mass

Individual fish body mass was not tracked over the exposure period, therefore mass gain or loss over the course of the experiment was not calculated. All goldfish after the 7-day and the 14-day exposures weighed statistically the same across exposure groups. Other studies, however, have observed weight gain in fluoxetine-exposed teleost such as hybrid striped bass, fathead minnow and goldfish [2,5,29]. Potential reasons for the difference in results are dose and duration of fluoxetine exposure, seasonal variability of growth in teleost fish and sex.

Some of the fluoxetine studies that observed differences in body mass gain were using much higher concentrations of fluoxetine in their exposures, such as 50 μg/L[29], or were exposing organisms for a longer duration (28 days[5]) than our study. One experiment observed a concentration and duration-dependent decrease in prey capture ability in hybrid striped bass following fluoxetine exposure[2], which may reflect the lack of change in our short-term, low dose exposure. However, the observed trend ($p=0.054$) of decreased body mass in fluoxetine-exposed male goldfish in the 7-day exposure was not present in the 14-day experiment, indicating duration of the exposure is not the only independent variable.

Teleost fish often vary in mass gain over the year due to the seasonal variability of growth hormones throughout the year. Goldfish, for example, gain less mass during their

reproductive periods in the spring and grow much faster during the late summer in preparation for winter [112]. When goldfish are in their rapid growth phase, any changes to their metabolism and growth would have a greater impact on their growth than if the effect had been during their reproductive phase. The lack of body mass difference in our 14-day exposure that took place in May may reflect the fact that the male goldfish were likely to be in their reproductive phase and were not expected to differ much in growth over a one week period. Average male GSI in the control group during the 14-day exposure was 4%, which coincides with previous reports of spawning season and male goldfish GSI[112]. The effect of fluoxetine on male mass decrease may therefore be subject to seasonal variability.

Sex of the animal may also affect the variability of fluoxetine-induced decrease in mass. While fluoxetine did not affect the body mass of the exposure groups, there was a significant interaction between sex and exposure. Males were significantly heavier than females in the control group, but this distinction was lost in fluoxetine exposed groups. The loss of difference in mass between sexes potentially indicates that males were losing more mass than females in the fluoxetine exposed groups compared to controls. Sex was not considered in the study by Gaworecki and Klaine[2], who observed reduced feeding in hybrid striped bass following fluoxetine exposure and sex was unable to be determined in the study by Stanley et al.[29], where fluoxetine affected feeding and growth of juvenile fathead minnows. Mennigen et al.[5] only considered females during their fluoxetine-affected feeding study. Sex, therefore, may be another important factor in fluoxetine-induced weight loss. Ultimately, the lack of difference between body mass between exposure groups and control is most likely due to the low dose and short duration of the fluoxetine exposure, with seasonality and sex potentially playing a partial role.

2.4.2. Short-term fluoxetine exposure affects male goldfish GSI

Gonadosomatic index reflects the reproductive phase of an animal and is often evaluated in EDC exposures to gain a rough estimate of potential EDC-induced reproductive effects. In the 7-day fluoxetine exposure, fluoxetine did not significantly change the male or female GSIs when compared to controls. These findings agree with those from several other studies that found no difference in various teleost fish species GSI following waterborne fluoxetine exposure[3,31,34,36]. This evidence supports the concept that fluoxetine does not modify gonad size relative to body size in teleost fish.

In contrast with the 7-day goldfish GSI results, the 14-day low dose fluoxetine exposure resulted in a significant decrease in male goldfish GSI compare to controls. Like goldfish growth, reproduction is highly seasonal, with the reproductive axis principally active in the late spring[113]. The effects of fluoxetine on goldfish GSI, therefore, may also be seasonal. The significant effect on GSI in our males exposed in May supports the concept that in a reproductive time, males may be more responsive to reproductive disruption and may experience more severe physiological effects than males exposed at less critical periods. The study by Mennigen et al.[3] study on fluoxetine-affected male goldfish reproduction took place in May and a separate fluoxetine exposure using sexually mature male goldfish took place in April[114]. Neither study showed a difference in GSI in the fluoxetine exposure groups. The 25% decrease in male GSI observed in our study is therefore worth pursuing in a differing dose time-course fluoxetine exposure to determine amount and duration of dosage, seasonal variability and sex effects of fluoxetine exposure on GSI.

While other studies did not find fluoxetine-induced change in fish GSI, other reproductive endpoints in teleost fish are affected by fluoxetine exposures. Waterborne

fluoxetine exposure decreases milt production and circulating testosterone levels[3] and induced hepatic vitellogenin mRNA production[114] in sexually mature male goldfish, decreases female zebrafish egg production and ovarian aromatase activity[36] and induces vitellogenin production in male fathead minnows[31]. This discrepancy between GSI data and other reproductive endpoints indicates that GSI is not a sensitive endpoint in identifying reproductive disruption caused by fluoxetine exposure and should be interpreted cautiously.

2.4.3. Short-term fluoxetine exposure increases CRF mRNA abundance in female goldfish brain

Out of the all of neuropeptides studied, only CRF mRNA was significantly changed by fluoxetine exposure in female goldfish, indicating that CRF may be one of the first feeding neuropeptides to be altered by SSRI actions in the brain. Consistent with our findings, Mennigen et al.[5] found similar increases in CRF mRNA in the hypothalamus of female goldfish following a 14-day waterborne fluoxetine exposure. Another study that considered CRF in the context of 5-HT signalling found similar results, in that 5-HT signalling impacted CRF abundance and locomotor actions in the brain of juvenile Chinook salmon (*Oncorhynchus tshawytscha*)[115]. Corticotropin-releasing factor is at least partially controlled by serotonergic neurons in the male rat brain and an increase in 5-HT levels in the pre-synaptic neurons enhanced CRF production[116]. Fluoxetine injections increase extracellular 5-HT levels in male rat brains[117] and thus may be increasing CRF mRNA in our female goldfish brain tissue through this mechanism.

Corticotropin-releasing factor neurons interacts to control NPY, CART-1 and urotensin-1 producing neurons to ultimately cause a decrease in fish appetite[15]. An injection study on rainbow trout identified CRF, urotensin-1 and 5-HT as anorexigenic agents and suggested that 5-HT-induced anorexia may be at least partially mediated by CRF-secreting neurons [16]. Ultimately, our CRF mRNA results suggest that the anorexigenic effects of fluoxetine may be mediated

through an increase in CRF early on, which may relate to decreased body mass after a more prolonged exposure in goldfish feeding and weight gain as found by Mennigen et al.[5].

The isotocin mRNA results provided new information to what has been previously established in the literature regarding the effect of fluoxetine on isotocin mRNA abundance in the teleost brain. Isotocin is the homolog to mammalian oxytocin and is known for its role in reproduction in the teleost system. In this study, isotocin was investigated as anorexigenic neuropeptide due to its interaction with ACTH and its association with reduced feeding in stressed and subordinate fish[118]. Isotocin detected by HPLC-fluorescence detection in gilthead sea bream (*Sparus auratus*) pituitary increased following food deprivation[119], however, suggesting an activation of synthesis by their storage in pituitary in response to food deprivation.

In previous research, fluoxetine injections reduced isotocin mRNA levels in sexually regressed female goldfish in the hypothalamus and telencephalon[37]. A 14-day waterborne fluoxetine exposure reduced isotocin expression in male goldfish telencephalon when exposed to a female pheromone at a pharmacological fluoxetine concentration (54 μg/L)[5], furthering the concept that isotocin and fluoxetine exposure are linked. In male rats, a 14-day fluoxetine injection treatment attenuated oxytocin responses to a selective 5-HT_{1A} receptor agonist[120]. In a separate study, a 21-day fluoxetine injection treatment in male rats attenuated 5-HT_{2A} receptor-mediated stimulation of oxytocin [121]. These studies similarly promoted the idea that isotocin and oxytocin signals in the brain are attenuated in fluoxetine-exposed animals.

In our fluoxetine study, a low dose of fluoxetine did not change isotocin mRNA abundance in male and female goldfish hypothalamus and telencephalon, similar to the findings of the 14-day waterborne exposure of Mennigen et al.[3]. However, isotocin mRNA abundance increased 3-fold (though not significantly) in male goldfish telencephalon in the high exposure

group in the present study. This was the first time the dose of 1 μg/L fluoxetine was studied and one of the two studies that shows a potential increase in isotocin mRNA following fluoxetine exposure. A 14-day waterborne fluoxetine exposure (33 μg/L) increased isotocin mRNA levels in male zebrafish whole brain, which matched the observed decrease in appetite[39].

The differences in the effects of fluoxetine on isotocin mRNA levels between species, sex, dose and mode of transportation into the system (injection or waterborne exposure) clearly indicate a complex connection between SSRIs, 5-HT, isotocin, feeding and stress. Similar to our CRF mRNA results, our isotocin results should motivate further investigations on the seasonality of isotocin and fluoxetine action in goldfish, with focus on dose, duration and mode of transportation of fluoxetine into the model.

The other neuropeptides investigated showed no significant differences in mRNA abundance between exposure groups, however, their role in the feeding disruptions observed in fluoxetine-exposed animals should not be disregarded. Conflicting results still exist in the literature regarding the effect of fluoxetine on NPY. One study found a decrease in goldfish hypothalamic NPY mRNA levels following fluoxetine exposure, while another found increases in NPY expression in zebrafish whole brain[5,39]. In rats, fluoxetine administration reduced hypothalamic NPY levels[14]. In our investigation, no significant changes in NPY mRNA were noted following the 7-day fluoxetine exposure in either sex or tissue. The lack of NPY effect is most likely due to the low level of fluoxetine used and the short exposure period, but may also indicate that NPY is not the primary neuropeptide altered by fluoxetine exposure.

Transcript abundance of CART-1 did not significantly change following the fluoxetine exposure. Administration of CART leads to decreases in NPY and orexin-A orexigenic actions[122], therefore, CART-1 is most likely not the main route of the effects of fluoxetine on appetite, as

major decreases in both NPY and orexin-A mRNA would have been expected. Secretogranin-IIa and CCK mRNA were similarly unchanged, indicating that their role in initial fluoxetine-induced appetite loss may be minimal.

Overall, fluoxetine did not disturb orexigenic neuropeptides after a short-term waterborne exposure, while it significantly increased one anorexigenic neuropeptide mRNA, CRF, in the female brain. These findings indicate that the main pathway that fluoxetine alters feeding motivation may be through stimulating anorexiogenic signalling rather than inhibiting orexigenic signals. In this regard, the effect of SSRIs may seem to be acting more intensely or more quickly to CRF and potentially isotocin neurons in the female brain. The modifications in NPY and CART-1 mRNA seen by other fluoxetine exposure studies may reflect secondary and indirect effects of fluoxetine from CRF and isotocin actions. The sex differences observed in this exposure also warrant further investigation.

2.4.4. Short-term fluoxetine exposure increases goldfish hepatic miRNAs

All of the hepatic miRNA investigated significantly increased in expression following fluoxetine exposure (with sex, dose and time-related differences in abundance levels). The miRNAs investigated in this experiment may therefore be epigenetic regulators in the cascade of fluoxetine effects. In zebrafish, the mRNA targets of these miRNA were predicted to be involved in cholesterol synthesis, insulin signalling and triacylglyceride synthesis[6]. One of the miRNA, miR-22, has also been found to play important roles in insulin signalling in other mammalian and fish species such as human (*Homo sapiens*), Japanese medaka, stickleback (*Gasterosteus aculeatus*), Fugu pufferfish (*Takifugu rubripes*) and zebrafish[123]. In humans, over-expression of *hsa-miRNA-22* is linked to increased insulin-stimulated Akt phosphorylation in the liver. In other fish species, miR-22 is also predicted to target components of the insulin pathway by targeting

phosphoinositide 3-kinase (PI3K) subunits. As such, it is very possible that the miRNA identified in Craig et al. microarray[6] may be partially responsible for the decreases in insulin signalling in the liver caused by SSRI exposure[5], demonstrating the potential role for miRNA as regulatory molecules in fluoxetine-induced disruption of normal liver function.

How fluoxetine exerts its effect on hepatic miRNA is still unknown. One proposed mechanism for miRNA control is through the manipulation of its biosynthesis pathway, by either regulating transcription or by modifying biosynthesis enzymes so miRNA is not cleaved properly. Within the cell, transcription factors such as SMAD proteins and the effectors of transforming growth factor-β/bone morphogenetic protein have been shown to up-regulate miRNA abundance by enhancing the cleavage activity of Drosha in the nucleus[124]. A recent review by Gupta et al. suggested that estrogen actively controls miRNA production in mammary and ovarian cells by manipulating miRNA transcription[50]. Estrogens inactivate RNA polymerase II in these cells and inhibit pre-miRNA biogenesis by blocking Drosha-mediated processing, thus down-regulating specific miRNA and ultimately leading to changes in ovulation.

Similarly, fluoxetine may be acting on specific miRNAs by altering their transcription or enzymatic processing. Fluoxetine lowered levels of miR-16 in serotonergic raphe neurons of mice by promoting the translation of the neurotrophic factor S100b in the raphe nucleus[66]. This neurotrophic factor acted as a paracrine signal to inhibit miR-16 production in both the locus coeruleus and the hippocampus in mice, ultimately leading to an increase in SERT expression in these areas. A potential cascade of fluoxetine-miRNA-protein signalling may also be how hepatic miRNA are being controlled by fluoxetine in goldfish.

The effect of fluoxetine on hepatic miRNA differed between males and females in our 7-day exposure, with female miRNA abundance increasing by a greater factor than males after

only 7 days of fluoxetine exposure. This difference in response between sexes is similar to the difference in responses observed in the neuropeptide mRNA levels and reflects the specific effect an EDC can have on separate sexes. In addition to the sex differences noted, there was also a dose-response to the nominal fluoxetine concentrations observed in the female hepatic miRNA, in that two of the miRNAs (*dre-miR-301a* and *dre-miR-457b*) studied showed a step-wise increase in response to higher doses of fluoxetine. This is the first time that a dose effect has been observed and indicates a potential dose-response that warrants further investigation.

A temporal effect was also observed in our male goldfish, as more miRNA were significantly increased following the 14 day fluoxetine exposure compared to the males exposed for only 7 days. These results must be interpreted cautiously, however, due to the difference in season at which the samples were taken. The males that responded strongly were responding during their peak reproduction phase (May), while those that did not significantly responded were in their growth phase (August). Rather than a temporal effect, that may be another example of a seasonal difference between our males, as previously discussed in sections 2.4.1-2.4.3.

The final steps in this study are to predict the mRNA targets in our goldfish models to determine if the effect of fluoxetine-induced metabolism upset may be conserved through miRNA action across species. Unlike zebrafish, which have a well annotated transcriptome, goldfish genomes and transcriptomes are less documented. In an attempt to identify mRNA targets in goldfish, RNA from various goldfish tissues (liver, brain, pituitary) was extracted and transcriptomes were sequenced by HiSeq. As future steps, we will assemble the transcriptome. While it is often difficult to adequately assemble 3-prime UTRs with initial RNASeq data, this is the critical first step to predict potential mRNA targets. By matching the predicted goldfish

mRNA targets with those found by Craig et al.[6], we may demonstrate support for miRNA as a strong candidate involved in metabolism upset caused by fluoxetine exposure.

2.5. Conclusions

We have demonstrated that short-term waterborne fluoxetine exposure increases CRF mRNA in female goldfish hypothalamus and telencephalon. This effect was not observed in male goldfish brain, indicating a sex-difference in the effect of fluoxetine on both the stress and feeding axis of goldfish. The change observed in isotocin mRNA, while not statistically significant, raised further questions regarding seasonal variations in the response to fluoxetine in terms of reproductive and stress systems. The lack of change in the other neuropeptide mRNA studied indicates that they may not be the primary peptides behind disruptions in feeding motivation in goldfish. We have also demonstrated that fluoxetine up-regulates several hepatic miRNAs that may be associated with metabolic pathways such as adipogenesis, and insulin signalling in teleost fish. These findings compliment the initial discovery of Craig et al.[6] and outline miRNA as a potential target for SSRIs and other EDCs in the environment.

Together, the brain and liver findings may complement each other. Female goldfish experienced increases in CRF mRNA levels in the brain as well as increases in hepatic miRNA following fluoxetine exposure, providing a potential link between CRF and liver function. While CRF action was initially thought to be restricted to the pituitary and brain, in humans it has also been expressed in peripheral tissues such as adrenals, testis, placenta, intestines, spleen, thymus, skin, pancreas, ovaries and heart[125]. Several studies have also observed expression of CRF receptors at the liver in humans, rats and mice, suggesting that CRF may play a role in the sympathetic regulation of hepatic pathophysiology. Based on our results, future research should focus on the potential link between CRF and liver function by attempting to detect CRF or

ACTH receptors in fish liver tissues and link the effect of fluoxetine to their stimulation. The brain and liver results from the two short-term fluoxetine exposures on adult goldfish provide a more detailed understanding of the feeding and metabolism disruption effects caused by fluoxetine exposure.

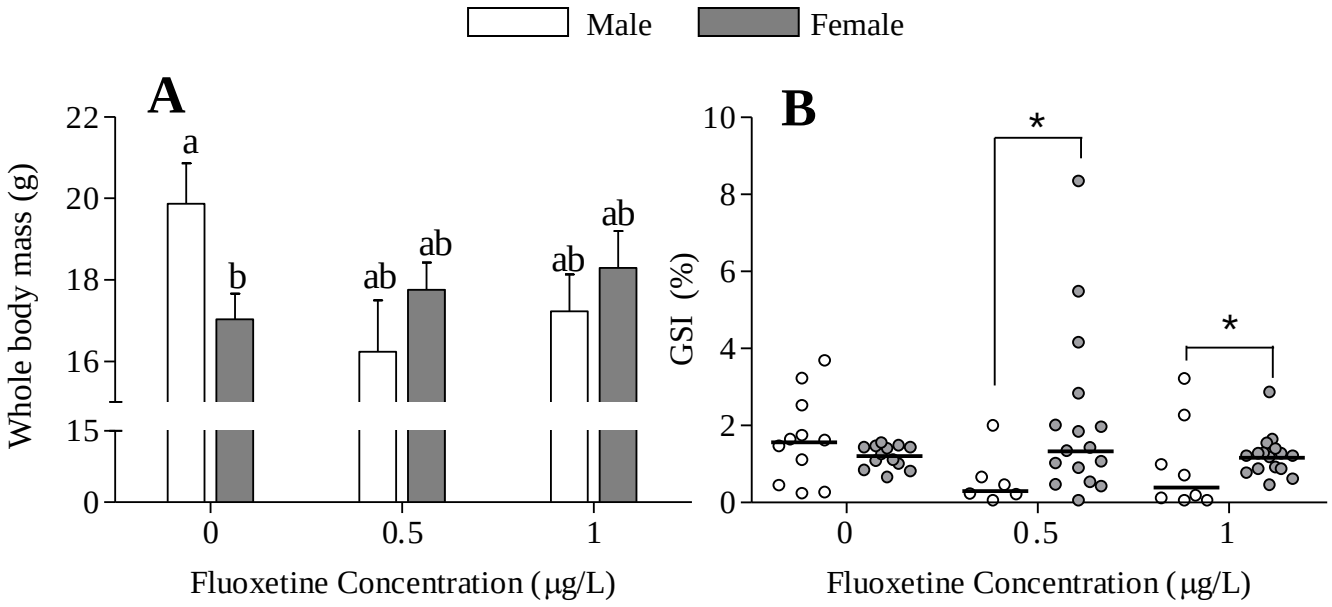

Figure 2.1. (A) Mean + SEM of male (n = 7-12)and female (n = 12-17) goldfish whole body mass (g) and (B) medians of male and female gonadosomatic index (GSI, %) in three waterborne exposure groups with 0, 0.5, and 1 µg/L fluoxetine following a 7-day exposure. Different letters (a,b) indicate significant differences between means, while * indicate differences between log10 normalized means (two-way ANOVA, Bonferroni post-hoc, $p<0.05$).

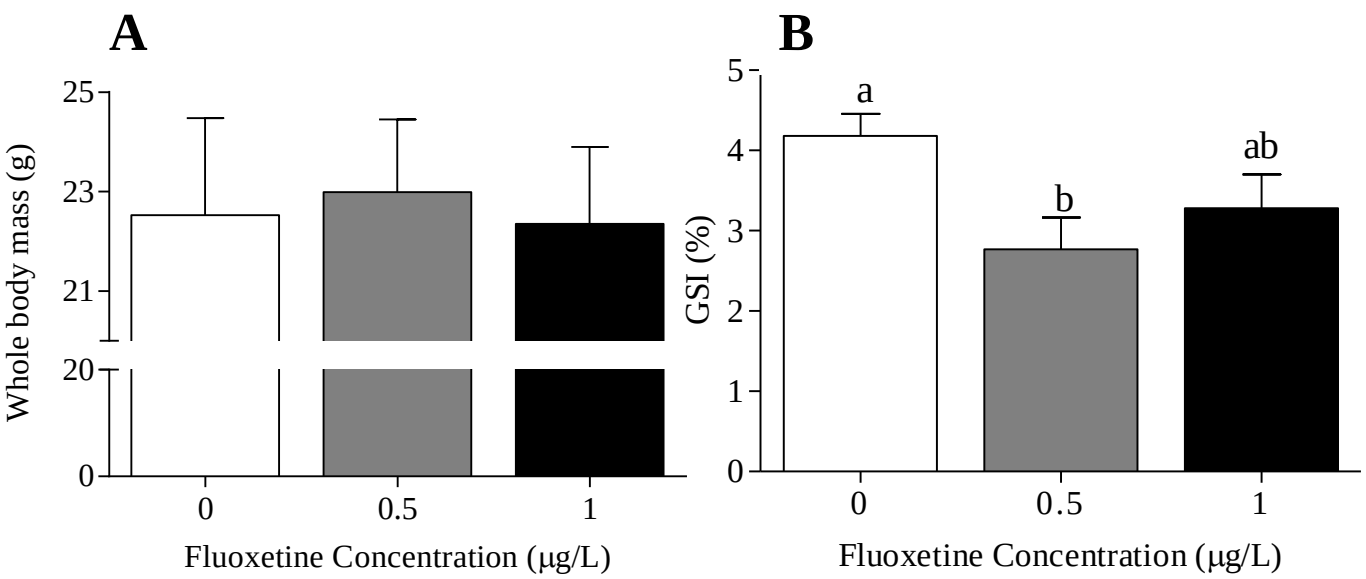

Figure 2.2. Mean + SEM of male goldfish (n = 9) (A) whole body mass (g) and (B) gonadosomatic index (GSI, %) in three waterborne exposure groups with 0, 0.5, and 1 μg/L fluoxetine following a 14-day exposure. Different letters (a,b) indicate significant differences between group means (one-way ANOVA, Bonferroni post-hoc, $p<0.05$).

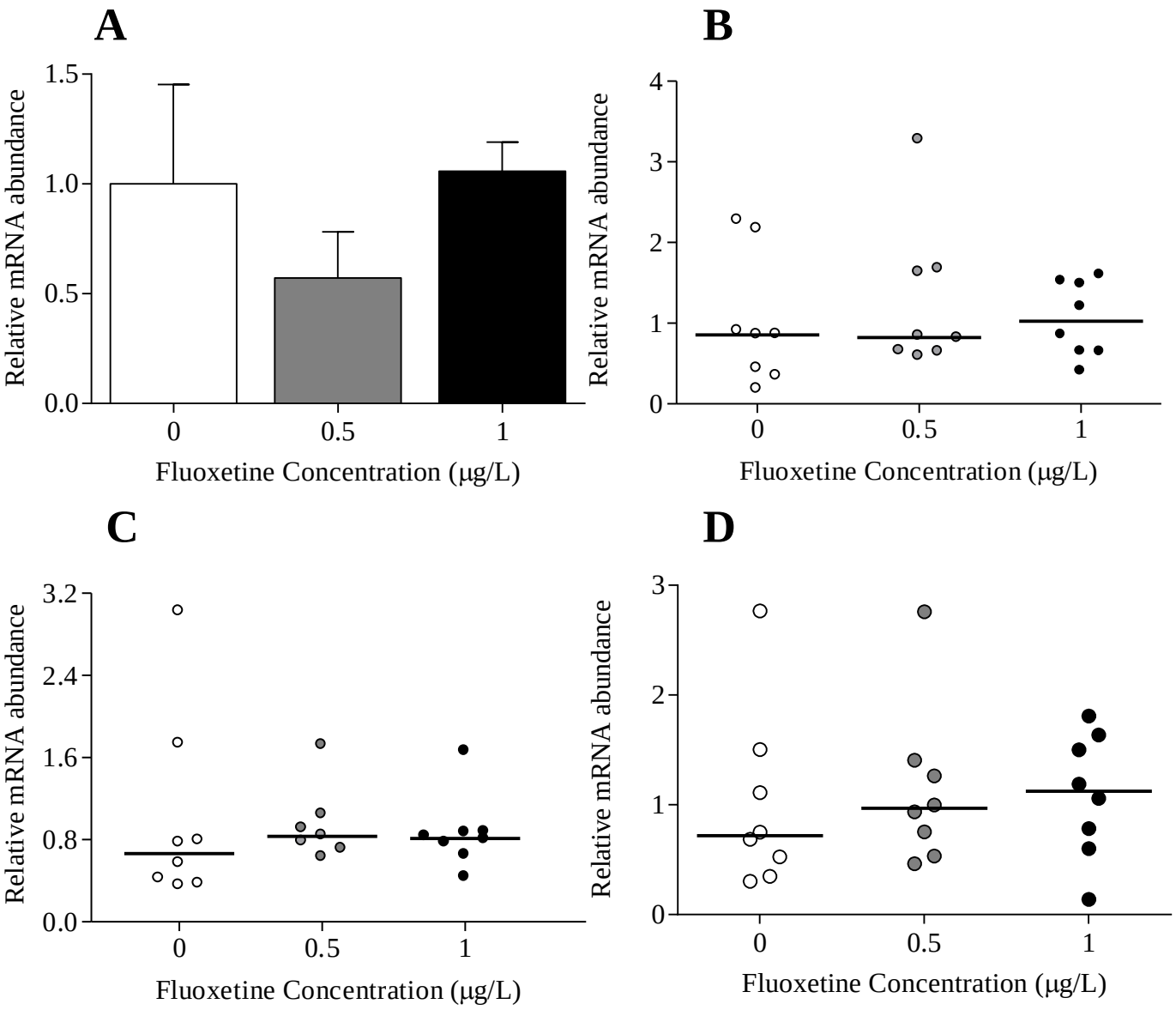

Figure 2.3. (A) Mean + SEM of relative mRNA abundance of neuropeptide Y (NPY) in male goldfish hypothalamus (n = 5), medians of relative abundance of NPY mRNA in (B) female goldfish (n = 8) hypothalamus, (C) male goldfish telencephalon (n = 8) and (D) female goldfish telencephalon (n = 8) tissue following a 7-day waterborne exposure to 0, 0.5, and 1 μg/L fluoxetine. No significant differences were observed between groups (one-way ANOVA).

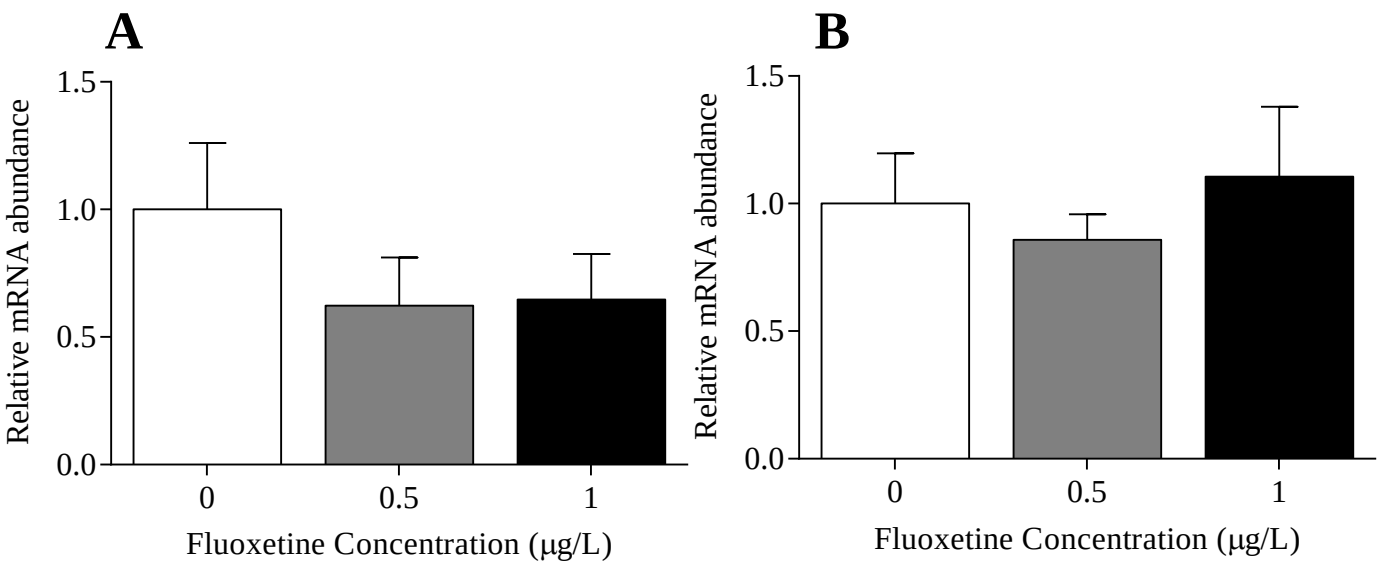

Figure 2.4. Means + SEM of relative mRNA abundance of orexin-A in (A) male (n = 5) and (B) female (n = 8) goldfish hypothalamus tissue following a 7-day waterborne exposure to 0, 0.5, and 1 μg/L fluoxetine. No significant differences were observed between groups (one-way ANOVA).

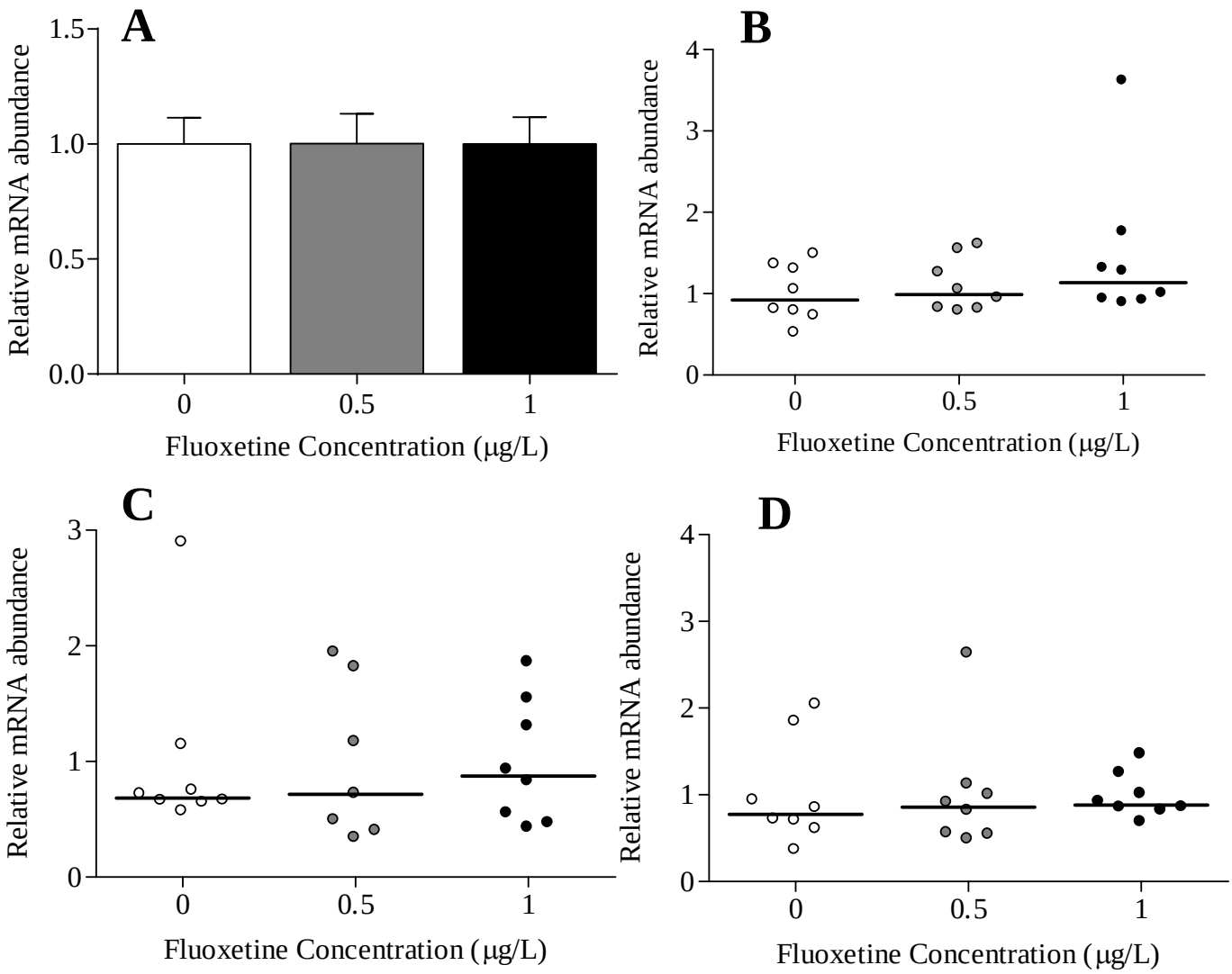

Figure 2.5. (A) Mean + SEM of relative mRNA abundance of secretogranin-IIa (SgIIa) in male goldfish hypothalamus (n = 5), medians of relative mRNA abundance of SgIIa in (B) female goldfish hypothalamus (n = 8), (C) male goldfish telencephalon (n = 8) and (D) female goldfish telencephalon tissue (n=8) following a 7-day waterborne exposure to 0, 0.5, and 1 μg/L fluoxetine. No significant differences were observed between groups (one-way ANOVA).

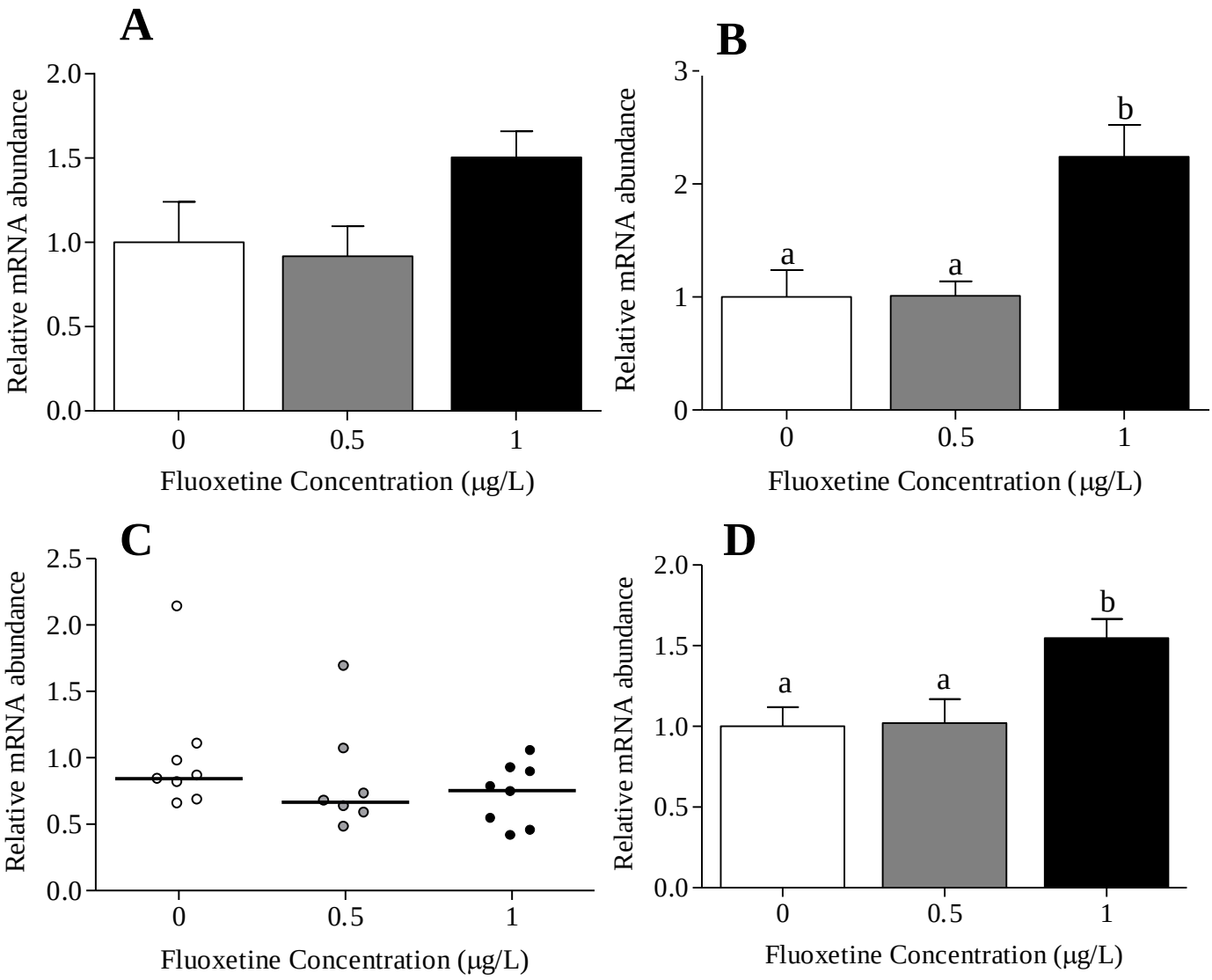

Figure 2.6. Means + SEM of relative mRNA abundance of corticotropin-releasing factor (CRF) in (A) male (n = 5) and (B) female (n = 8) goldfish hypothalamus, medians of relative mRNA abundance of CRF in (C) male goldfish telencephalon (n = 8) and (D) means of relative mRNA abundance of CRF in female goldfish telencephalon tissue (n = 8) following a 7-day waterborne exposure to 0, 0.5, and 1 μg/L fluoxetine. Different letters (a,b) indicate significant differences between groups (one-way ANOVA, Tukey post-hoc, $p<0.05$).

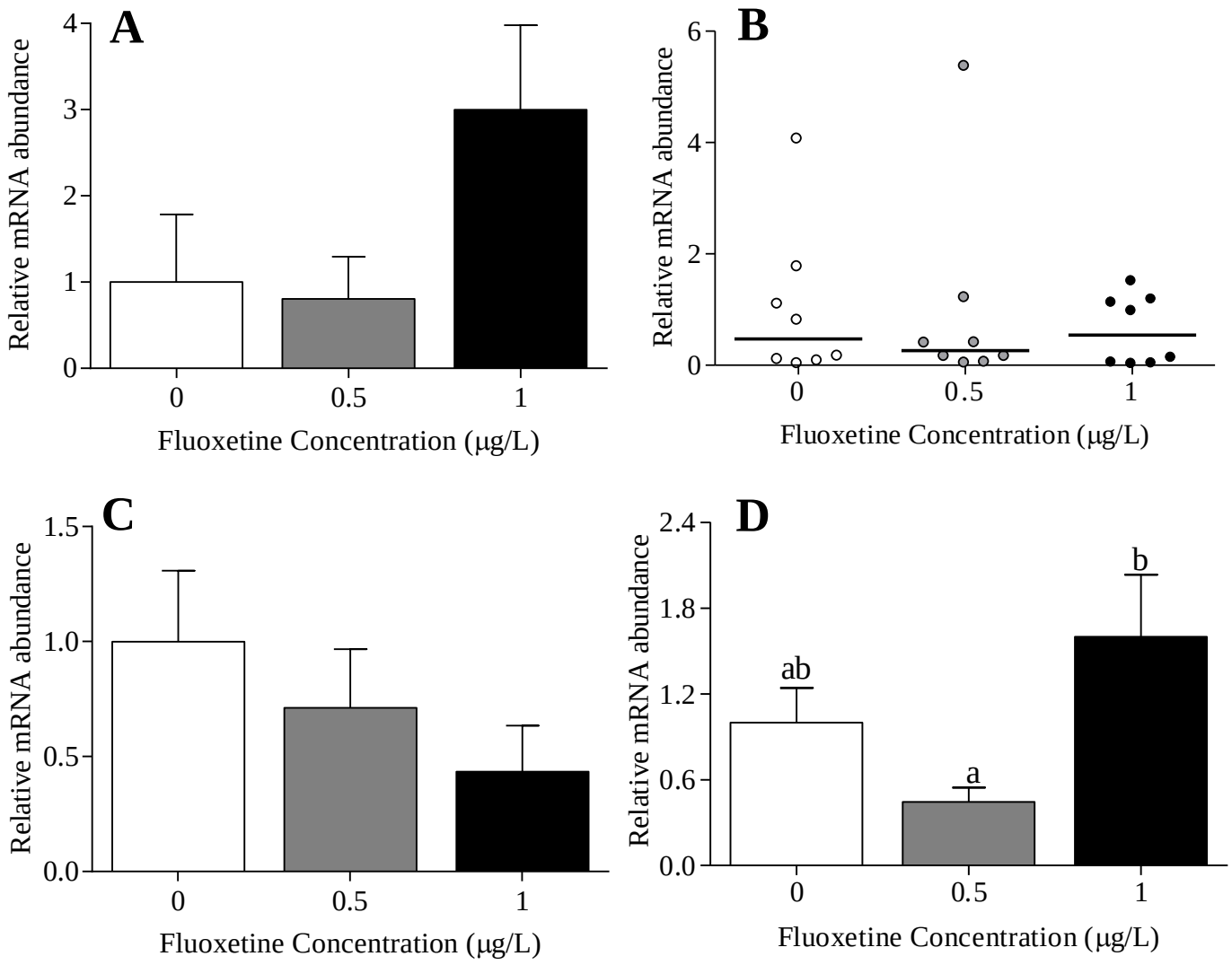

Figure 2.7. (A) Mean + SEM of relative mRNA abundance of isotocin in male goldfish (n = 5) and (B) medians of relative abundance of isotocin mRNA in female goldfish (n = 8) hypothalamus , and means of relative mRNA abundance of isotocin in male t (n = 7) and female goldfish telencephalon (n = 8) tissue following a 7-day waterborne exposure to 0, 0.5, and 1 μg/L fluoxetine. Different letters indicate significant differences between means (one-way ANOVA, Tukey post-hoc, $p<0.05$).

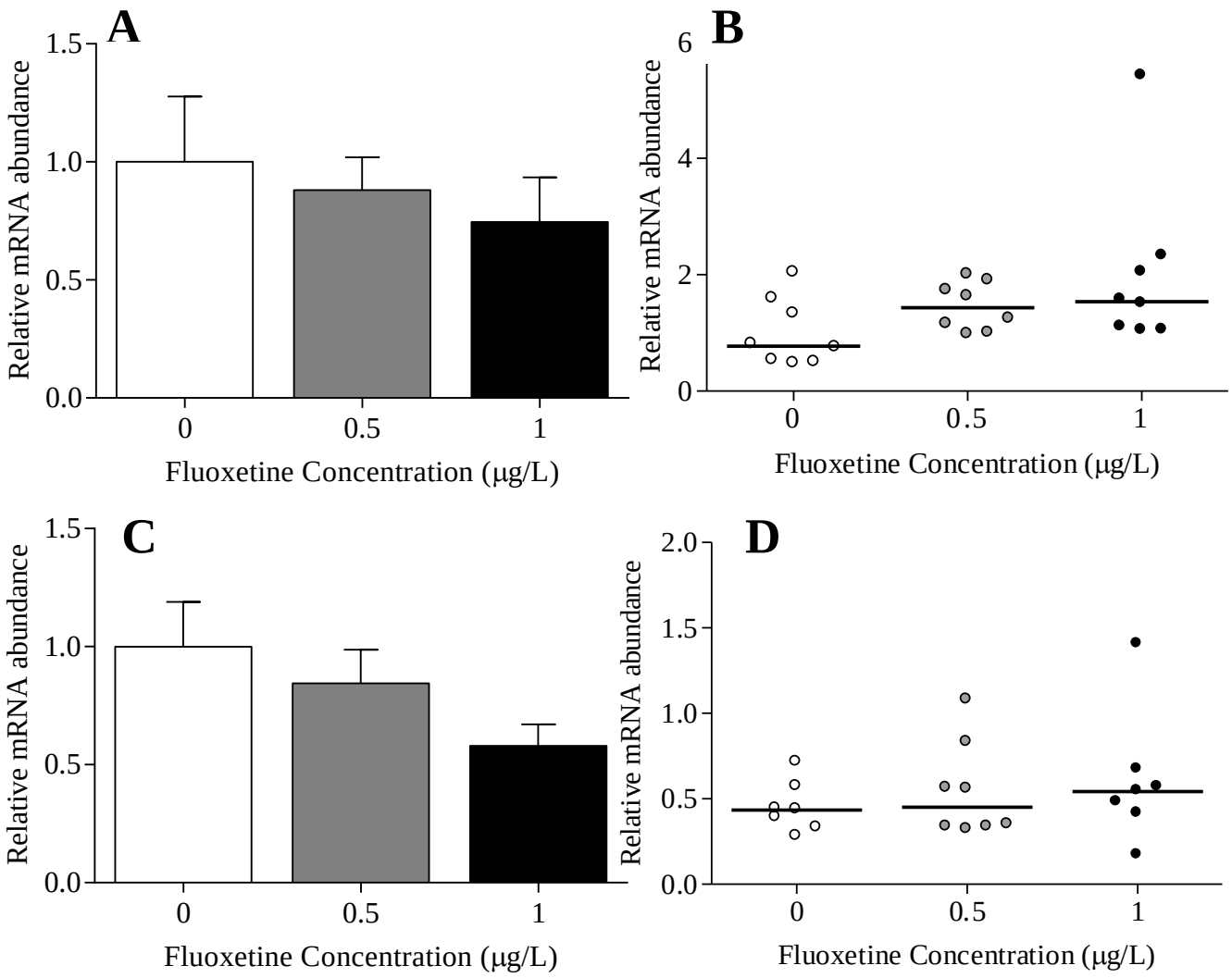

Figure 2.8. (A) Mean + SEM of relative mRNA abundance of cholecystokinin (CCK) in male goldfish hypothalamus (n = 5), (B) medians of relative mRNA abundance of CCK in female goldfish (hypothalamus n = 8), (C) mean + SEM of relative mRNA abundance of CCK in male goldfish telencephalon (n = 7) and (D) medians of relative mRNA abundance of CCK in female goldfish telencephalon (n = 8) following a 7-day waterborne exposure to 0, 0.5, and 1 µg/L fluoxetine. No significant differences were observed between groups (one-way ANOVA).

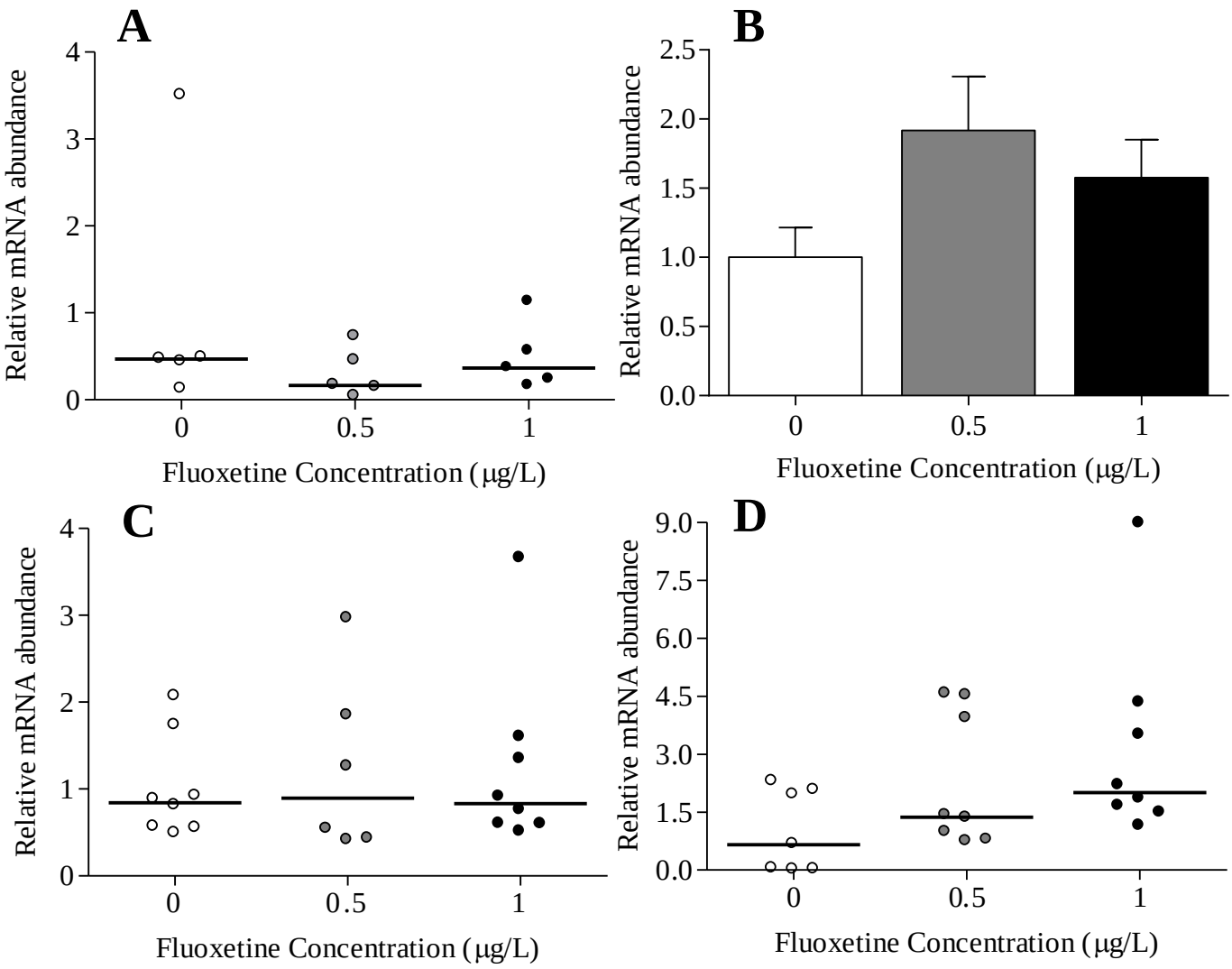

Figure 2.9. (A) Medians of relative mRNA abundance of cocaine- and amphetamine-related transcript-1 (CART-1) in male goldfish hypothalamus (n = 5), (B) mean + SEM of relative mRNA abundance of CART-1 in female goldfish hypothalamus (n = 8), and medians of relative mRNA abundance of CART-1 in (D) male (n = 7) and (D) female goldfish telencephalon following a 7-day waterborne exposure to 0, 0.5, and 1 μg/L fluoxetine. No significant differences were observed between groups (one-way ANOVA).

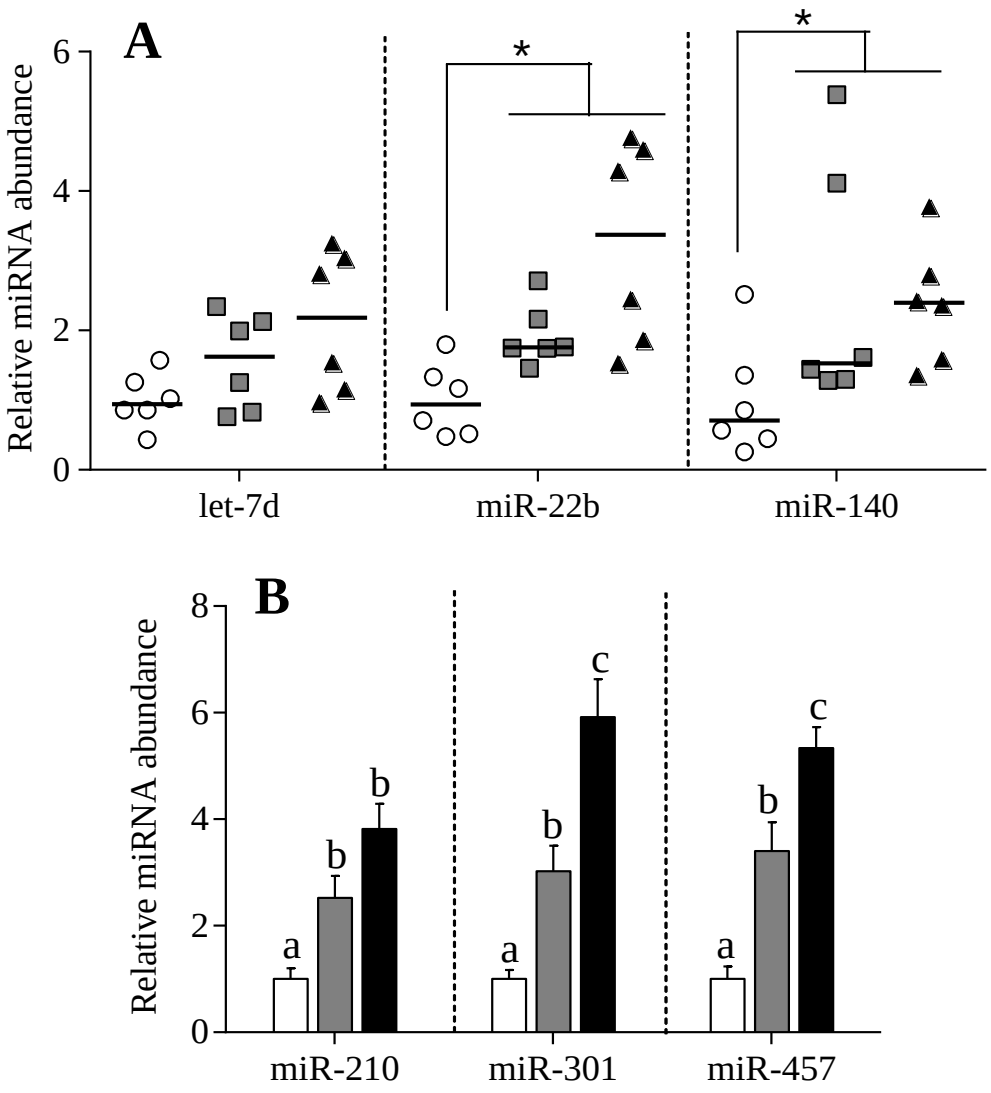

Figure 2.10. (A) Medians of relative miRNA abundance in female goldfish (n=6) liver tissue of *dre-let-7d*, *dre-miR-22b*, *dre-miR-140-5p*, and (B) means + SEM of *dre-miR-210*, *dre-miR-301a*, and *dre-miR-457b-5p* following a 7-day waterborne exposure to 0 (white), 0.5 (grey), and 1(black) µg/L fluoxetine (FLX). Different letters (a,b) indicate significant differences between means, while * indicate differences between log10 normalized means (one-way ANOVA, Tukey post-hoc, $p<0.05$).

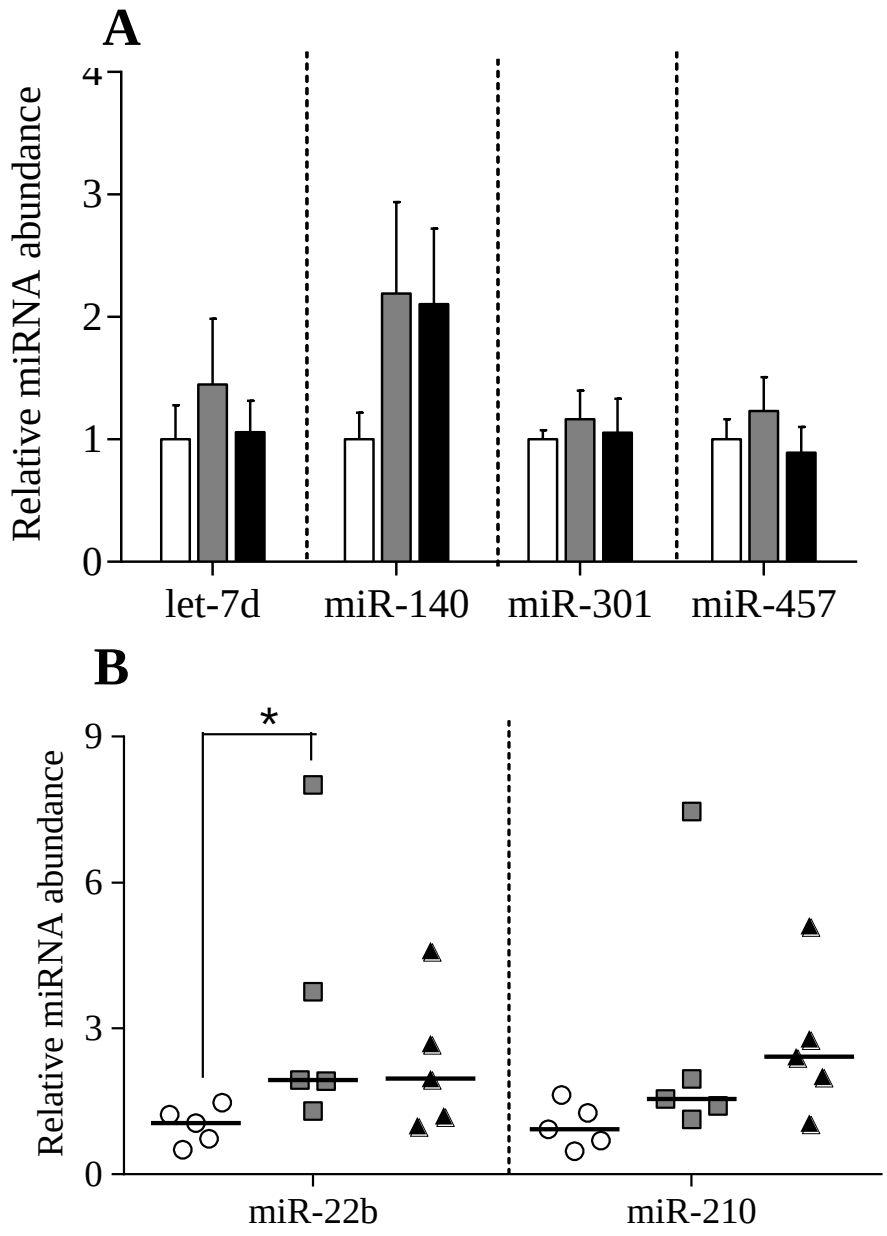

Figure 2.11. (A) Means + SEM of relative miRNA abundance in male goldfish (n=5) liver tissue of *dre-let-7d, , dre-miR-140-5p, dre-miR-301a,* and *dre-miR-457b-5p* and (B) medians of *dre-miR-22b* and *dre-miR-210* following a 7-day waterborne exposure to 0 (white), 0.5 (grey), and 1 (black) µg/L fluoxetine (FLX). * indicates differences between log10 normalized means (one-way ANOVA, Tukey's post-hoc, $p<0.05$).

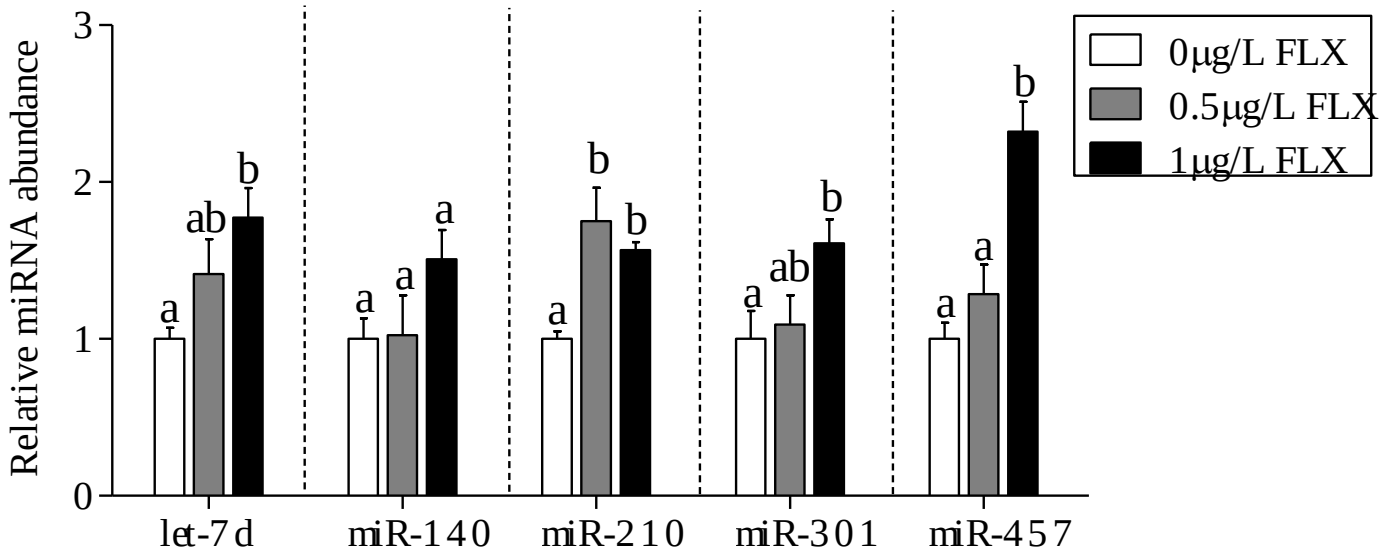

Figure 2.12. Means + SEM of relative miRNA abundance in male goldfish (n=4) liver tissue of *dre-let-7d*, *dre-miR-140-5p*, *dre-miR-210*, *dre-miR-301a*, and *dre-miR-457b-5p* following a 14-day waterborne exposure to 0, 0.5, and 1 µg/L fluoxetine (FLX). Different letters (a,b) indicate significant differences between means, while * indicate differences between log10 normalized means (one-way ANOVA, Tukey's post-hoc, $p<0.05$).

CHAPTER 3:

The effects of short-term waterborne fluoxetine exposures on serum and bile metabolite profiles of *Carassius auratus*

3.1 Introduction

Fluoxetine, the active ingredient in the antidepressant Prozac ®, is found in our aquatic environment at concentrations capable of causing energy metabolism disruption in exposed fish[2,5,6,126]. Fluoxetine is known to act at both the brain and liver of exposed fish to cause changes in feeding neuropeptide mRNA (goldfish)[37] and hepatic miRNA (goldfish, zebrafish)[6] levels, which coincide with reduction in feeding and liver metabolism disruption caused by fluoxetine exposures (goldfish, striped bass)[2,5]. A serum proteome study also discovered several peptides involved in diabetes mellitus that were increased in the serum of fluoxetine-exposed male goldfish[114], demonstrating a link between fluoxetine exposure and disrupted liver function that was observable in the serum of exposed fish.

To consider all of the potential downstream effects that fluoxetine may have on metabolic activity, this experiment attempts an exploratory metabolomic approach. Metabolomics is the emerging field in systems biology that provides an overview of the metabolic status of a cell, tissue, or organism and reflects the phenotype of an animal in a given condition at a given time. By evaluating the small molecule (100-1500 Daltons in size) changes following waterborne fluoxetine exposure in goldfish serum and bile, this study aims to discover metabolites that may confirm previous findings regarding metabolism disruption and uncover unknown disrupted metabolic pathways by using a non-targeted approach.

Adult goldfish were exposed to fluoxetine in two short-term exposures (14- and 7-day) at environmentally relevant concentrations[23,99] (0.5 and 1 μg/L) and blood and bile metabolite

profiles were examined. Serum was selected as a biofluid to investigate due to its unique role as a transporter of chemicals to and from other tissues in the body. Serum metabolite profiles represent the physiology of an animal by containing the end products of multiple tissues and are often used in metabolomic investigations. For example, serum metabolomics is being increasingly used in the medical field for diagnosis of kidney and liver dysfunction[127]. In addition, if this metabolomic approach is to be applied, a non-invasive extraction method is the most suitable to measure biomarkers in wild fish. Fish bile was selected for analysis as biliary excretion is a major removal mechanism for many environmental contaminants and is therefore useful in assessing the exposure of xenobiotic compounds from water[128]. Since bile is produced by the liver to excrete liver waste products, it is also useful in considering the downstream effects on liver function following fluoxetine exposure. The detection of metabolites can also be more feasible, as regular physiological functions naturally bioconcentrate contaminants in the bile prior to excretion. A study that used solid phase microextraction coupled with LC tandem MS found that fluoxetine bioconcentrated in fathead minnow bile following exposure to a various fluoxetine exposures (3.2- 320 μg/L) [128]. By comparing the serum and bile metabolite profiles of fluoxetine-exposed and unexposed goldfish, an unbiased and robust evaluation of phenotypic changes in metabolic function can be accomplished.

Ultra performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-Q-TOF) was used to separate and detect small molecules present in the bile and serum for each exposure. Principal Component Analysis (PCA) and Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA) were used to statistically identify discriminating metabolites in each biofluid, which could then be tentatively identified using online databases. Due to the large variability of most metabolites across all goldfish samples, a smaller selection of

metabolites was assembled and used to reveal any subtle fluoxetine-induced changes in both biofluids. Cluster analysis and canonical discriminant analysis were then used to individually determine any significant differences between exposure groups. This fluoxetine metabolomic investigation is one of the first of its kind in the field of ecotoxicology. Based on these novel findings, future metabolomic investigations can become more efficient and effective at providing potential new pathways in which EDCs exert their effects on exposed organisms.

3.2 Materials and methods

3.2.1. Experimental design

See section 2.2.1 for animal handling, experimental design and tissue collection for the 7-day and 14-day waterborne fluoxetine exposures.

3.2.2. Serum and bile sample preparation for UPLC-Q-TOF

Due to the relatively new application of metabolomics in endocrine disruption investigations, there was no standardized sample preparation procedure available in the literature for the analysis of goldfish serum or bile using the UPLC-Q-TOF (Xevo-G2, Waters Corporation, Milford, MA, USA). Consequently, proof-of-principal and sample preparation method development for UPLC-Q-TOF analysis were needed. Before selecting the final sample preparation protocol, the serum and bile profiles of males and female goldfish were compared using different extraction procedures in an effort to find the most efficient and effective extraction method that provided the best signal to noise ratio, separation and isotopic fit of metabolites on the UPLC-Q-TOF and encompassed as many small and relevant molecules as possible in each sample.

The selection of the reported protocol was based on the ease of preparation, minimal extraction steps required and relatively abundant, strong signal intensities for metabolite profiles.

While 75% acetonitrile (ACN) was the solvent of choice for metabolite extraction, several other combinations of solvents were also attempted such as methanol, 50:50 methanol:ACN and 2% ACN, and various dilution factors (1:5-1:10000) were considered due to other metabolomic studies that successfully used these combinations[82,129,130]. In several attempts, solutions were dried down with a nitrogen stream and reconstituted in ACN and methanol before being filtered. Ultimately, several of the methods were complimentary, each providing a similar yet unique profile.

In the finalized protocol, 100 μL of each serum sample and 20 μL of each bile sample were used for analysis. An equal volume of 75% ACN (in LC/MS grade H_2O) was added to each serum sample and 10 fold the bile volume was added to the bile samples, followed by a brief vortex of 5 seconds. Samples were then kept on ice for 30 minutes before being centrifuged at 4°C and 14,600 rpm for 15 minutes. Pellets containing proteins were discarded and 100 μL of the remaining supernatants from each sample were added to 100 μL 75% ACN and vortexed for 5 seconds. After 30 minutes on ice, the samples were centrifuged with the previous parameters. This process was repeated to ensure complete removal of protein and resulted in a total dilution of 1:8 and 1:100 for serum and bile, respectively. Samples were then filtered using a 0.22 μm syringe filters, where 50 uL of sample was placed in the filter tip and pushed through the filter into high recovery injection vials. This process was repeated until 300 μL of each sample was filtered. Samples were then frozen at -80°C before being thawed on ice, sonicated and analyzed.

3.2.3. UPLC-Q-TOF conditions

Ultra performance-LC analysis was performed on a Waters Acquity UPLC equipped with an Acquity UPLC® BEH C18 column (2.1 mm × 50 mm, i.d. 1.7 μm, Waters Corp, Milford, USA). The analytical column was maintained at a temperature of 50 °C and the mobile phases

was composed of ACN (A) and water (B) each containing 0.1% formic acid. A solvent gradient system was used: 5-100% A for 15 min with a flow rate of 0.8 mL/min. Injection volume was 1 μL for both exposures.

Quadrupole-TOF analysis was performed in both positive and negative ion modes. Positive ion mode was used for analysis due to better signal to noise ratio and peak capacity. Data from the Q-TOF were collected in the full scan mode from m/z 100-1500. All the data were acquired using an independent reference lock mass via the LockSpray^{TM} interface to ensure accuracy and reproducibility during the Q-TOF analysis.

3.2.4. Statistical analysis

Following the UPLC-Q-TOF scan, the raw data was analyzed using MassLynx (V4.1 SCN918, Waters Corp). Metabolite signal intensities were detected within 0.25-5 min retention time window for 14-day and following improvement of separation methods, 0.25-8.5 min retention time window for 7-day exposure. Marker intensity threshold was set at 1000 counts within a mass window of 0.05 and noise elimination level of 8. The intensity of each ion was normalized with respect to the total ion count to generate a data matrix.

Marker Lynx software (MassLynx, V4.1, SCN918, Waters Inc.) was used for multivariate analysis to investigate the differences in metabolite signal intensity between samples. Principal component analysis provided a 2D scores plot which plots the two principal components that encompass the most variance within the data set as the x and y axes, providing a visual overview of all of the samples and their potential groupings. Following the visualization of grouped samples in the PCA, OPLS-DA was applied to identify discriminating metabolites between treatment groups. Any discriminating metabolites (as detected by MarkerLynx XS, 95% confidence interval) were then selected for further identification.

Finally, individual evaluation of the trend plots of each metabolite across all samples was performed to identify important metabolites that may have been missed by the multivariate statistical evaluation for each exposure. Serum and bile metabolites that showed visual increasing or decreasing signal intensity trends according to exposure group were selected for nonparametric cluster analysis and further canonical discriminate analysis.

Bile and serum clusters were separately generated using SAS software (V9.4 of the SAS System© 2014, SAS Institute Inc. Cary, NC, USA). MODECLUS, a nonparametric clustering procedure of SAS software, revealed the potential number of clusters that existed within the data set. The number of clusters was a function of the smoothing parameters, and the longest number of identical clusters irrespective of smoothing parameters indicated the most probable number of clusters in the dataset. Clusters were then compared using canonical discriminant analysis to confirm significant differences between groups. This nonparametric analysis was complimentary to PCA and revealed if the individuals in an exposure group cluster together with statistical significance.

3.2.5. Strategy for metabolite identification

This final step was not completed due to time and budget limitations; however, a strategy for metabolite identification is outlined here. The putative identity of a metabolite of interest is first predicted using the known retention time and sensitive mass of the metabolite as detected by Q-TOF. METLIN (metlin.scripps.edu/) is used to provide potential identification and molecular formulas for the metabolite in question. Based on molecular composition and probability, an assumption is made on the most likely identity of a metabolite. That chemical is then purchased and run through a tandem MS for confirmation. Once a metabolite is officially and concretely

identified, its exact concentration in the samples would be quantified by use of standard curve and QTRAP® analysis.

3.3. Results

3.3.1. Serum and bile metabolite profiles following 14-day fluoxetine exposure

Over 3000 metabolites were detected by Q-TOF in the serum and bile samples of the control and fluoxetine-exposed goldfish. Fig. 3.1 demonstrates a typical serum (Fig. 3.1A) and bile (Fig. 3.1B) metabolite profile of a control male goldfish.

3.3.1.1. PCA and OPLS-DA results

When evaluating all bile samples from all three treatment groups (0, 0.5, and 1 μg/L fluoxetine), the PCA score plot demonstrated that fluoxetine exposure was the first principal component, in that there was a separation of five of the eight bile samples from high exposure group from the rest of the samples (Fig. 3.2A). Using discriminant analysis, however, no discriminating metabolites were observed between control and high exposure bile groups (Fig. 3.2B). A similar lack of discriminating metabolites was observed for the low exposure bile sample and control bile samples (data not shown).

The PCA score plot for serum was not as distinct as that of the bile; however, fluoxetine exposure did appear to be the principal component separating the data (Fig. 3.3A). When comparing control serum samples to the high exposure serum samples in OPLS-DA, one metabolite was potentially discriminating in the high exposure group and was selected for further analysis (Fig. 3.3B). Serum samples from the low exposure group did not show any major distinguishing metabolites compared to control serum samples in OPLS-DA (data not shown). The trend plot for the selected serum biomarker is shown in Fig. 3.4.

3.3.1.2. Trend plot analysis of metabolites and subsequent cluster analysis

Following PCA and OPLS-DA, the trend plots for all 3062 metabolites detected in serum and bile samples were individually assessed. Any metabolites that showed an increasing or decreasing trend across exposure groups were selected for nonparametric cluster analysis. Using 39 bile metabolite signal intensities across all three treatments, cluster analysis revealed that the bile samples were clustering into either three or two groups (Fig. 3.5A). Two clusters were the most probable, as this number had the longest number of identical clusters across the k-values, irrespective of smoothing parameters. Upon further investigation of the samples in each group, five of the eight high exposure bile samples clustered outside of all the other bile data (table 3.1), similar to previous PCA findings. Using the 52 serum metabolite signal intensity data for cluster analysis, no grouping that reflected exposure was found, in that all of the samples clustered into 5 or 4 groups, irrespective of exposure or other notable physiological features (Fig. 3.5B).

Table 3.1. Cluster analyses of 22 goldfish bile samples following a 14-day waterborne fluoxetine exposure with three groups (control: no exposure, low: 0.5 μg/L fluoxetine, and high: 1 μg/L fluoxetine) Number of clusters stabilized at two between K-values of 5-7 based on 39 selected metabolites detected by UPLC-Q-TOF.

Cluster	**Number of bile samples from each exposure group**		
	Control	Low	High
1	8	8	3
2	0	0	5

3.3.1.3. Identification of OPLS-DA identified serum metabolite from 14-day fluoxetine exposure

While the discriminant analysis did not show any statistically discriminating metabolites for either serum or bile, one metabolite was selected for tentative identification due to its presence in all high exposure serum samples and absence in control and low exposure samples (detected at 80% confidence interval from OPLS-DA). The accurate molecular weight was

searched on METLIN, a comprehensive metabolite database, which provided tentative identification within 5 ppm mass accuracy threshold. Based on the molecular mass (310.1417 m/z) of the metabolite in the positive ion mode, the METLIN database search predicted the identity of the metabolite to be fluoxetine, to be confirmed by future analysis.

3.3.2. Serum and bile metabolite profiles following 7-day fluoxetine exposure

3.3.2.1. PCA and OPLS-DA results

For the analysis of the serum and bile metabolite profiles of male and female goldfish following the 7-day exposure, the PCA score plot showed two samples in the high-exposure group as potential outliers (data not shown). The metabolite profiles of these samples revealed unusually low signal intensity most likely due to significantly lower metabolite abundance, thus the samples were considered outliers and removed from the group. Principal component analysis and discriminant analysis for both bile and serum revealed no discriminant metabolites between any exposure groups (Fig. 3.6 and 3.7). No metabolites were therefore selected for identification based on multivariate analysis.

Individual evaluation of the trend plots of the 882 metabolites from all bile and serum samples provided a list of only 4 potentially discriminant metabolites in bile (Table 3.2) and 3 potentially significant metabolites in serum (Table 3.3) based on increasing or decreasing signal intensity trends across treatment groups. Due to time constraints, cluster analysis was not performed on 7-day exposure data.

Table 3.2. Retention times and molecular mass of manually selected goldfish bile metabolites following a 7-day fluoxetine exposure as detected by UPLC-Q-TOF.

Ret. Time (min)	Molecular mass (g/moL)
4.29	288.2890
0.69	296.0668
0.69	318.0476
5.77	475.3393

Table 3.3. Retention times and molecular mass of manually selected goldfish serum metabolites following a 7-day fluoxetine exposure as detected by UPLC-Q-TOF.

Ret. Time (min)	Molecular mass (g/moL)
7.65	180.0656
7.65	208.0970
0.69	229.1546

3.4 Discussion

For the first time, extraction methods for serum and bile metabolite profiles in the goldfish model species were established for UPLC-Q-TOF analysis. The developed extraction and analysis methods were able to detect up to 3000 small molecules above the noise level within 100-1500 m/z in both biofluids, providing a foundation for future work to investigate EDC exposure using the metabolomic approach.

3.4.1. Potential detection of fluoxetine in goldfish serum following 14-day exposure

Following the 14-day and 7-day fluoxetine exposures, multivariate analysis did not offer any discriminant metabolites that related to fluoxetine exposure in either serum or bile. However, there was potential detection of fluoxetine in the serum of goldfish exposed to the highest concentration after 14 days, demonstrating uptake of fluoxetine after two weeks of waterborne exposure. Other exposure studies have also found fluoxetine uptake and bioconcentration in fish following waterborne exposure at environmental levels[30,31]. Paterson and Metcalfe[30] exposed Japanese medaka to 0.64 μg/L fluoxetine for 7 days and found that within 5 hours, fluoxetine and norfluoxetine were detected in whole body fish tissues and both were found to bioconcentrate during the exposure, with BCF for fluoxetine ranging from 74 to 80. A separate study found fluoxetine and norfluoxetine in brain tissues of male fathead minnows during a 21-day waterborne exposure, also suggesting its bioconcentration in the brain[31]. Indeed, fluoxetine is likely to be found in the brain at levels several fold higher than in plasma. Fluoxetine was not

detected in the serum of goldfish exposed to the low dose, nor in the serum of fish exposed after the 7-day fluoxetine exposure. This discrepancy in detection may be due to the selection of serum as the analysed fluid following fluoxetine exposure. Due to its lipophilicity, fluoxetine has a large volume of distribution is more likely to bioconcentrate in fatty tissues such as brain and liver[21]. While serum sampling is relatively non-invasive and is useful in analysing wild populations and considering multiple organ metabolite products, it may not be the most appropriate tissue to detect fluoxetine at low exposure doses.

Still, the lack of fluoxetine detected in our 7-day and low dose exposures may also be due to the analytical techniques (non-targeted approach) used in our investigation, as fluoxetine and norfluoxetine have been detected in human plasma by targeted high performance-LC with ultraviolet detection within 6-8 hours of administration[131,132]. Ultimately, the potential discovery of fluoxetine in the serum of our high exposure goldfish demonstrates that, while limited, the untargeted approach is capable of detecting metabolites unique to EDC exposure.

3.4.2. Cluster analysis confirms PCA results for 14-day exposure bile samples

Interestingly, the detection of fluoxetine in the serum samples of the high exposure group goldfish following the 14-day exposure coincides with the PCA and cluster analysis results of the bile samples. The bile samples of the low exposure group clustered with the samples of the control group, while high exposure group bile samples clustered separately. These findings demonstrate that the higher nominal concentration of fluoxetine (1 μg/L) was more likely to cause downstream systemic effects, which were more likely to be observed in the samples of bile than serum. These exposure results coincide with the goldfish brain and liver data (Chapter 2), in which the high fluoxetine exposure caused neuropeptide mRNA and hepatic miRNA increases while the low doses remained relatively unchanged.

3.4.3. Selecting optimal parameters for metabolomic investigations of EDC exposure

Metabolite profiles are inherently dynamic, as they represent the constant changes in cellular actions in a tissue and are influenced by genetic and environmental factors. The magnitude of these influences varies and is difficult to measure as it can change over years, days, and even seconds within an organism. When analysing such dynamic profiles, variability within data is a large concern. Metabolite profiles of some tissues may be more variable than others, as we observed in our exposures. Serum is the most popular biofluid in the field of metabolomic research[127] due to its non-invasive extraction and its representation of circulating molecules in an organism, yet it is one of the most rapidly changing tissue in terms of metabolites. Even so, some studies are evaluating serum metabolite profiles following EDC exposure successfully. Sammuelson et al.[82] demonstrated that the discovery-driven approach of metabolomics was effective in demonstrating molecular changes in rainbow trout blood caused by prolonged waterborne 17α-ethinylestradiol (EE2) exposure. Proton NMR was used to detect blood EE2 biomarkers. For example, vitellogenin, phospholipids and cholesterol were identified in EE2 exposed male trout blood. These metabolomic findings coincided with the additional plasma vitellogenin determination by competitive enzyme-linked immunosorbent assay (ELISA) as well as previous EE2 exposure study results of vitellogenin production[133]. The coordination of metabolomic findings with ELISA results demonstrated that serum has potential for metabolomic investigations. In our study, the individual serum profile differences within exposure groups superseded any minor changes caused by the exposure and multivariate analysis could not detect any subtle differences.

In addition to tissue variability, it is limiting to only consider a single time point of a metabolite profile as a good representation of an animal's physiology as a result of an

environmental stressor. Time-course metabolomic evaluations allow for a baseline of what is considered "normal" for control organisms to be compared across exposure. Benskin et al.[134] collected hepatic metabolite profiles of sockeye salmon (*Onchorhychus nerka*) at different time points during in-migration and were able to observe natural changes in a subset of the hepatic metabolome and compare them to xenobiotic-exposed salmon at spawning time. Ekman et al.[86] profiled lipid metabolite responses of fathead minnows exposed to EE2 by taking liver samples throughout the exposure and were able to compare sex and time differences in the liver in response to exposure duration, providing a more complete picture of EE2 effects on the minnow hepatic lipid metabolome. In our exposures, all metabolite profiles were taken at the same time point at the end of the exposure, which limited our ability to distinguish natural fluctuations in metabolite profiles of goldfish from fluoxetine-induced metabolite changes. Further time points during our exposure may have provided a clearer image of "normal" goldfish metabolite profiles and a better distinction between fluoxetine-exposure and normal variability.

The low concentration and short exposure time of fluoxetine may have also attributed to of the lack of observable change in metabolite profiles by multivariate analysis. The environmentally relevant concentrations of fluoxetine used did not cause major changes in either serum or bile metabolite profiles of goldfish after short-term exposures using the multivariate analysis. Therapeutic responses to fluoxetine have also exhibited high individual variability in human patients[135]. In one metabolomic investigation, metabolite profiles of blood samples of juvenile male rhesus monkeys administered fluoxetine over a year period still did not separate completely in discriminant analysis, demonstrating that individual variability was a consistent finding even at such high doses and long duration of fluoxetine administration[135]. Trapp et al. [136] admit that while ideally a biomarker should be modulated only by contaminants, abiotic and

biotic factors influence the outcome of the exposure making it difficult to clearly discriminant a stressor-induced response from intrinsic variability. While there is also the possibility that fluoxetine causes no systemic effects, this can be rejected due to the abundance of literature suggesting otherwise[38,39,44]. Instead, it may be more likely that other abiotic and biotic influences rendered the non-targeted approach unable to detect fluoxetine-induced changes in the serum and bile metabolite profiles using multivariate analysis.

Another likely cause of the lack of observable effect following fluoxetine exposure may have been the equipment and statistical tools used. When comparing HPLC and H NMR use in metabolomic investigations, Wilson et al.[84] argued that most chromatographic methods that require the selection of columns and elution conditions can result in an unintended bias for this reason. Proton NMR is so much more likely to give stable results when the animals are so dynamic in response, due to its more robust detection methods. Other sources of variation in our data may include extraction methods and detection parameters used. Following these first attempts to determine the effects of fluoxetine on the goldfish metabolome, more proof-of-principal studies are required to improve exposure set-up, sample preparation and analysis methods.

3.4.4. Conclusions

This experiment was one of the first to attempt to use metabolic fingerprinting to evaluate fluoxetine effects in teleost fish. Fluoxetine was potentially detected in the high exposure group of our goldfish exposure to fluoxetine for 14 days, which related to the clustered bile samples of the high exposure group from the same 14-day experiment. These findings demonstrate that fluoxetine most likely caused a systemic change in the liver excretion of exposed organisms, but multivariate analysis was insufficient at identifying subtle differences. As such, the individually

selected metabolites may be worth identifying in future research and may potentially provide information on subtle excretion differences in fluoxetine-exposed fish. Overall, it seems as though bile is a more likely tissue to provide stable metabolite profile data and that the higher exposure levels and longer exposure durations are more likely to cause systemic changes observable in serum and bile. Future studies should also consider extracting metabolites from liver and brain tissues, which are known fluoxetine-targets, thus improving the likelihood of observing metabolomic differences.

In both fluoxetine exposures, the variability of signal intensities of individual metabolites was far too great across exposure groups for any other significant differences to be observed between treatments using the multivariate analysis. As such, no inferences regarding fluoxetine-induced phenotypic change were able to be made. Future studies that focus on the systemic effects of fluoxetine exposure in fish should consider carefully the exposure set up, sample preparation, and statistical analysis methods used when attempting to determine metabolite changes in dynamic fish tissues. Our findings demonstrate some of the challenges of determining potential changes in serum and bile of goldfish following fluoxetine exposure, while highlighting the importance of selecting the appropriate exposure set-up and statistical analysis method to properly analyze the large dataset of serum and bile metabolite profiles.

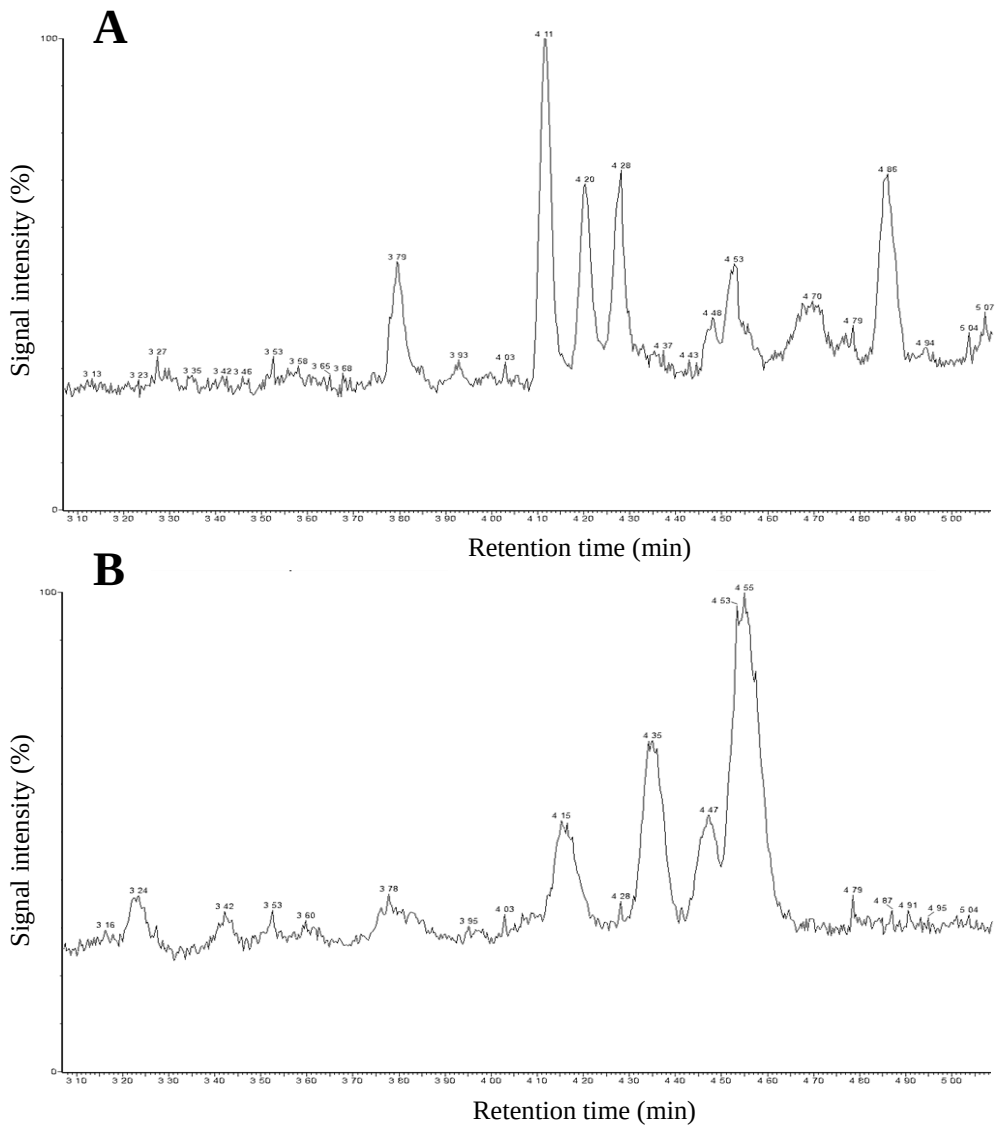

Figure 3.1. The retention time (min) and signal intensities (%) of approximately 1000 metabolites found in goldfish (A) serum and (B) bile as detected by UPLC-Q-TOF from 0-5.25 minutes detection time in positive ion mode. Signal intensity of a metabolite is displayed in relation to the highest intensity peak of the profile (1.26e5).

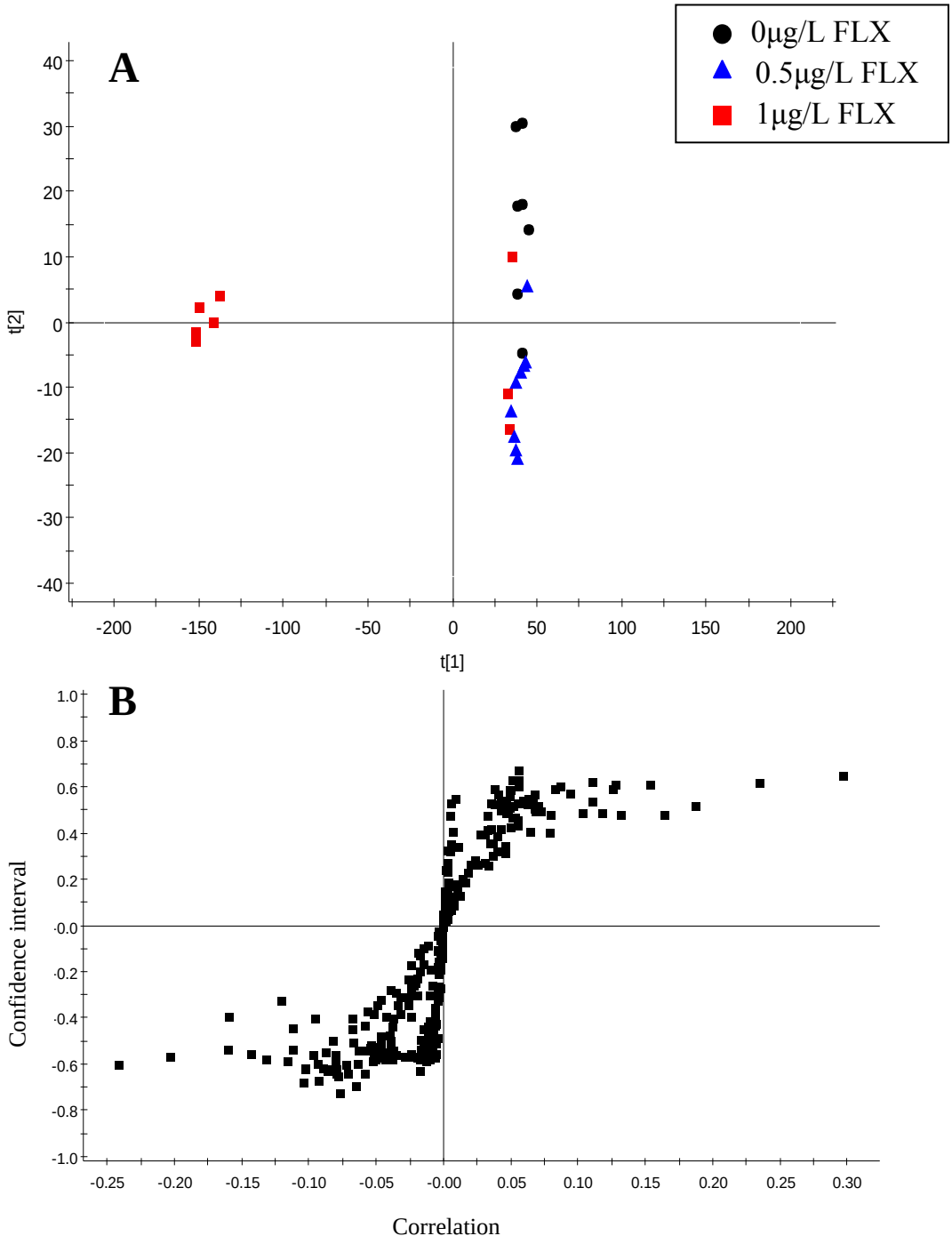

Figure 3.2. (A) PCA score plot of bile metabolite profiles of male goldfish (n = 24) from three fluoxetine (FLX) exposure groups (0, 0.5, and 1 µg/L) after a 14-day exposure period and (B) OPLS-DA S-plot comparing the metabolites of 0 and 1 µg/L fluoxetine exposure groups. For Panel A, X-axis is principal component 1 (t[1]) and y-axis is principal component 2 (t[2]). For panel B, X-axis is correlation of metabolites between exposure groups and y-axis is confidence interval. Tissue samples were extracted and diluted with 75% acetonitrile and were analysed using UPLC-Q-TOF.

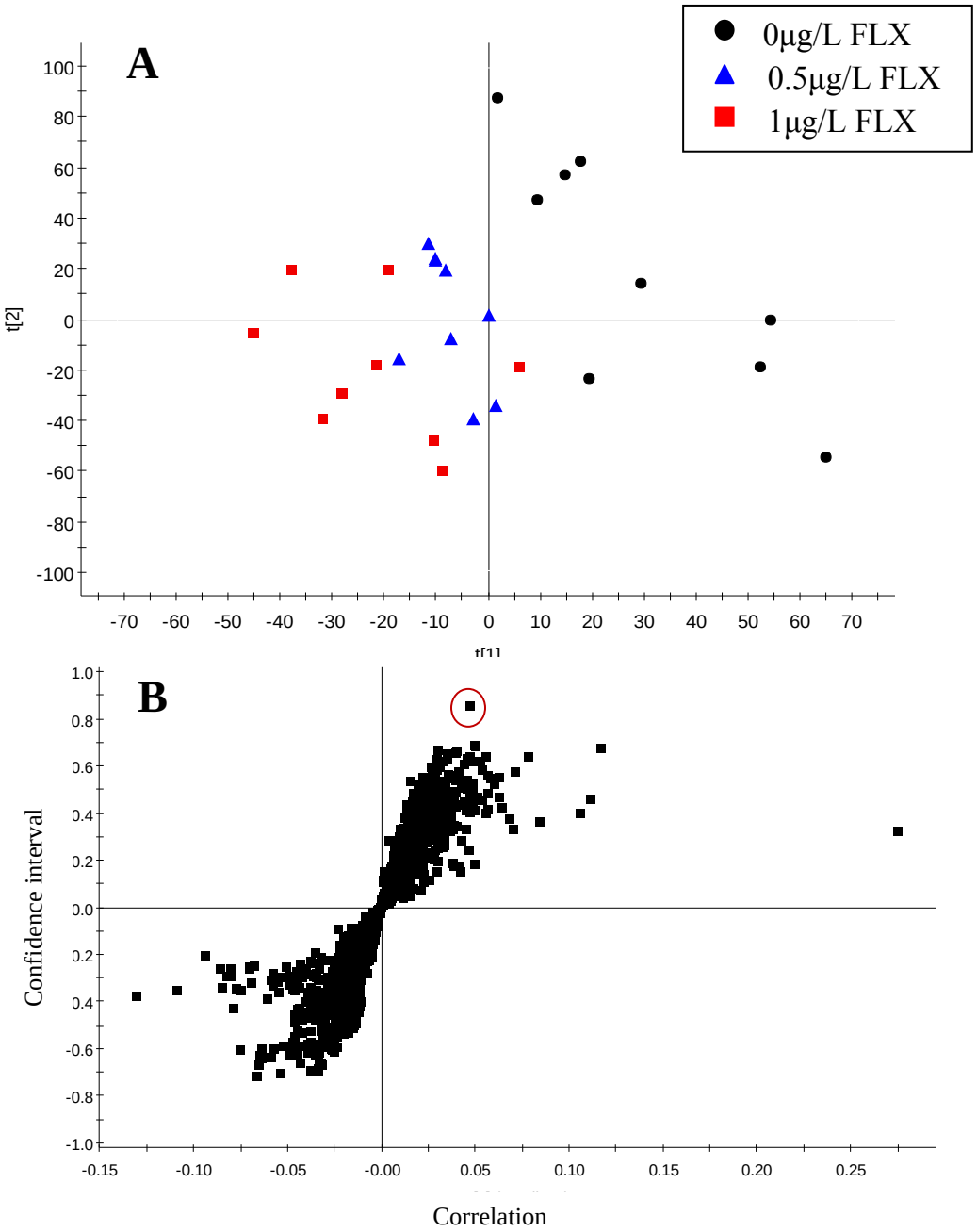

Figure 3.3. (A) PCA score plot of serum metabolite profiles of male goldfish (n = 27) from three fluoxetine (FLX) exposure groups (0, 0.5, and 1 µg/L) after a 14-day exposure period and (B) OPLS-DA S-plot comparing the metabolites of 0 and 1 µg/L fluoxetine exposure groups. For Panel A, X-axis is principal component 1 (t[1]) and y-axis is principal component 2 (t[2]). For panel B, X-axis is correlation of metabolites between exposure groups and y-axis is confidence interval. Tissue samples were extracted and diluted with 75% acetonitrile and were analysed using UPLC-Q-TOF. Potential metabolite of interest is circled in red.

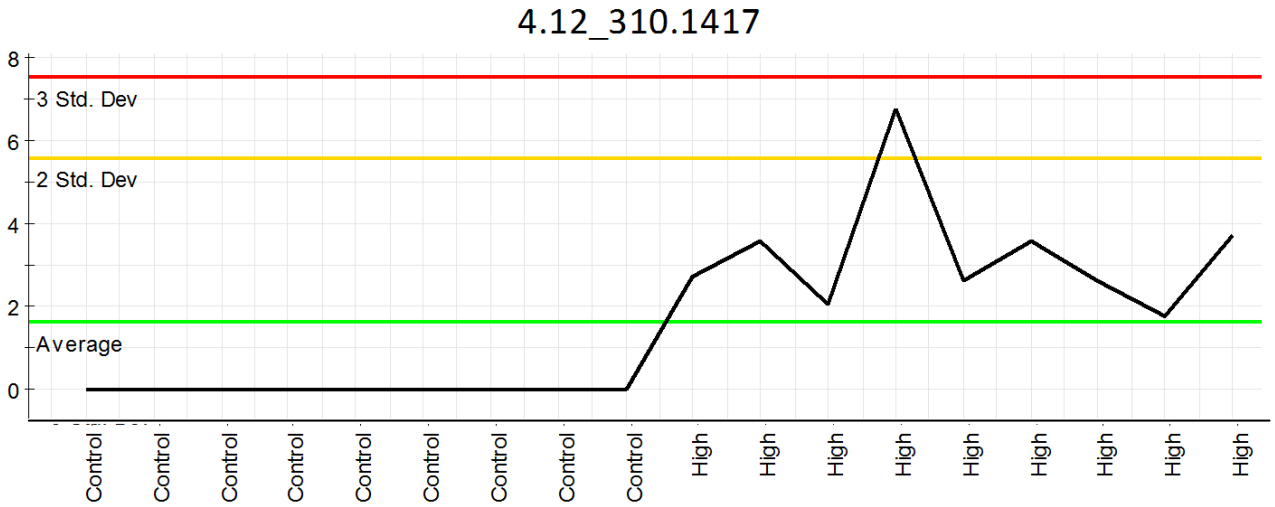

Figure 3.4. Trend plot of the signal intensities of the potential serum biomarker for fluoxetine in each sample (control= no treatment n = 9, high= 1µg/L fluoxetine for 14 days (n = 9)). Metabolite retention time (min) and molecular weight (m/z) in positive ion mode are indicated.

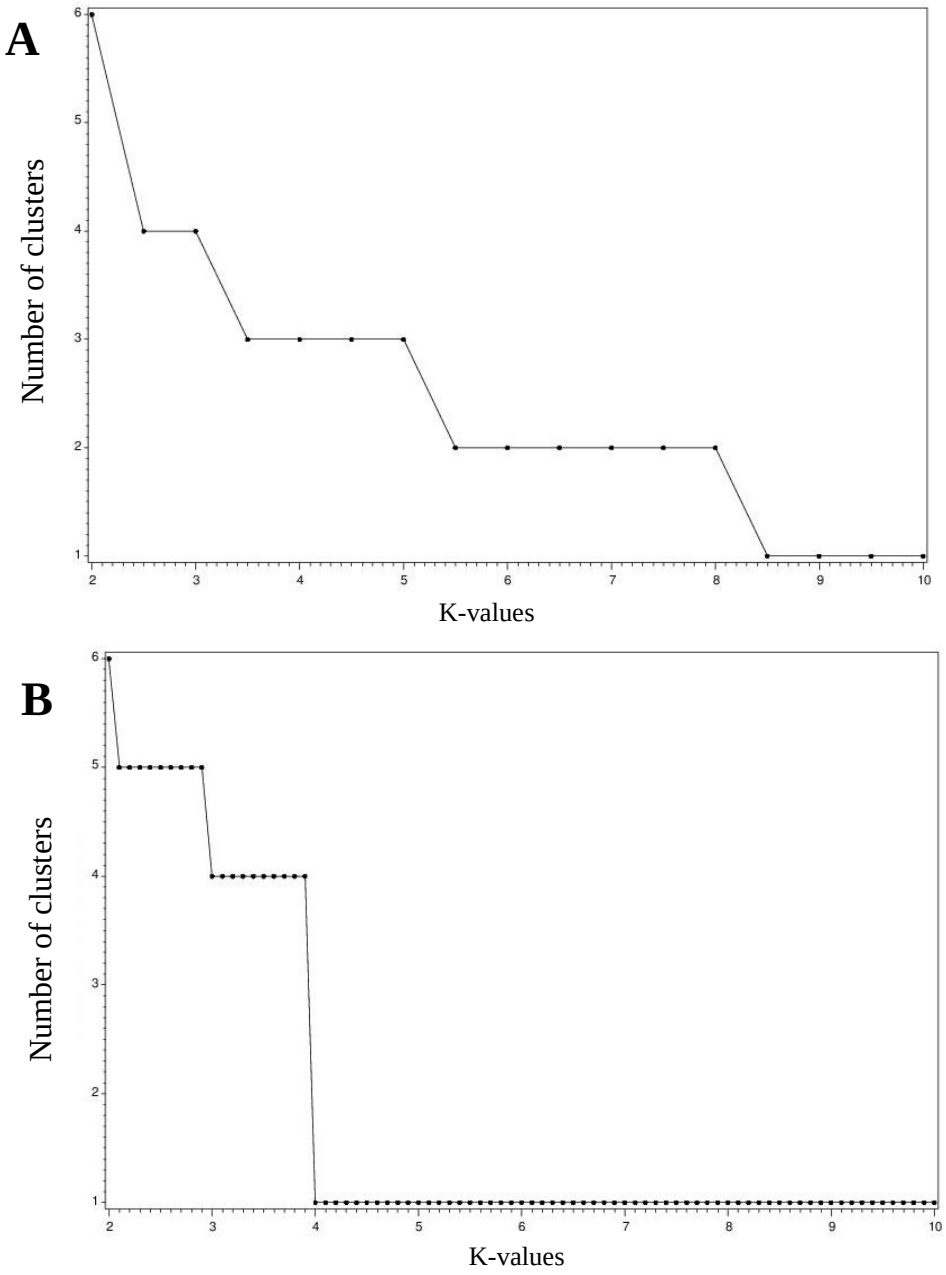

Figure 3.5. Cluster numbers as a function of k-values of (A) bile samples (n = 24) and (B) serum samples (n = 27) of goldfish following a 14-day fluoxetine exposure. Metabolite signal intensities by UPLC-Q-TOF were used to allow for clustering of samples into groups. Most likely number of clusters in a group is suggested by the longest number of identical clusters irrespective of smoothing parameters.

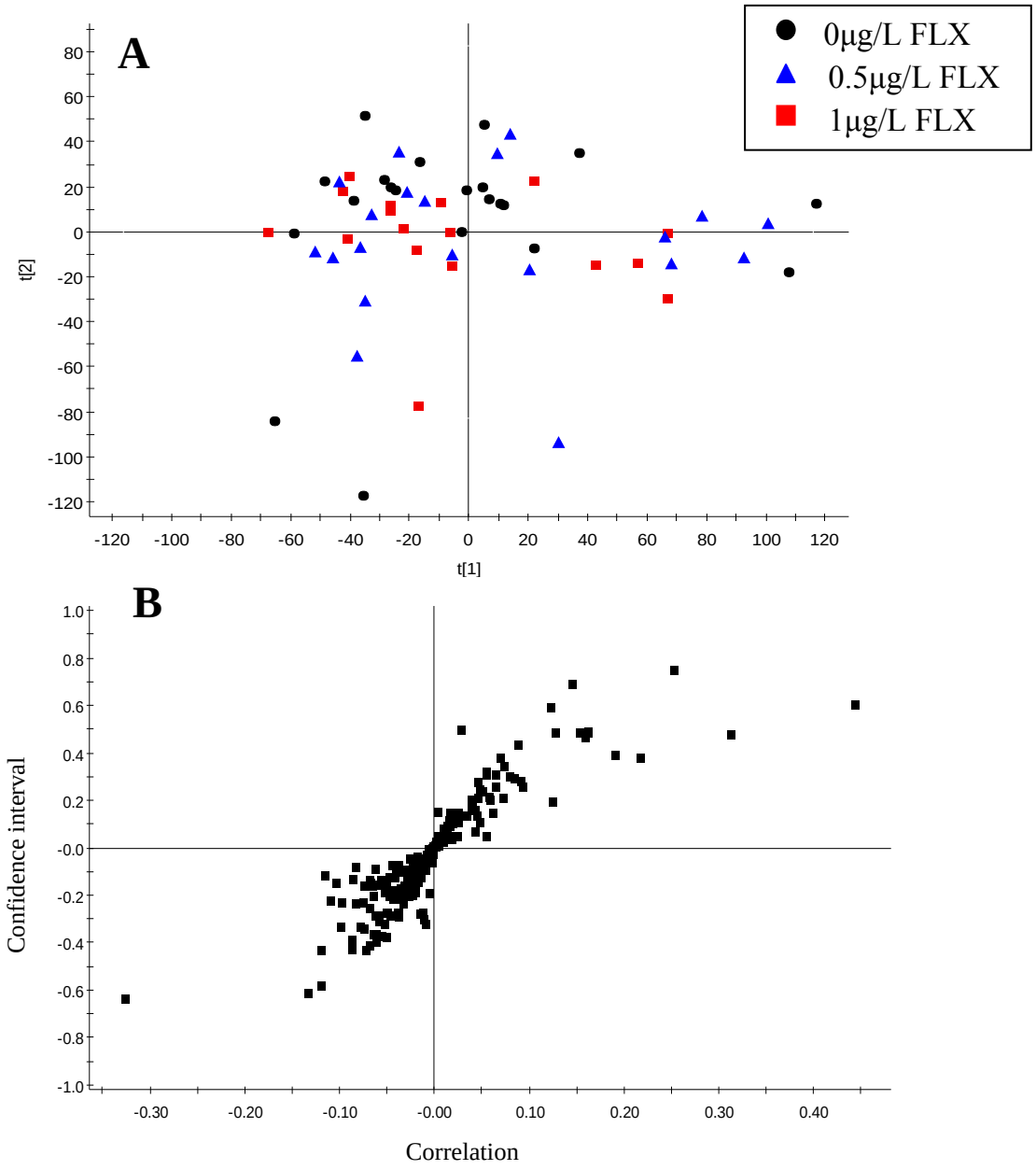

Figure 3.6. (A) PCA score plot of bile metabolite profiles of male and female goldfish (n = 70) from three fluoxetine (FLX) exposure groups (0, 0.5, and 1 µg/L) after a 7-day exposure and (B) OPLS-DA S-plot comparing the metabolites of 0 and 1 µg/L fluoxetine exposure groups. For Panel A, X-axis is principal component 1 (t[1]) and y-axis is principal component 2 (t[2]). For panel B, X-axis is correlation of metabolites between exposure groups and y-axis is confidence interval. Tissue samples were extracted and diluted with 75% acetonitrile and were analysed using UPLC-Q-TOF.

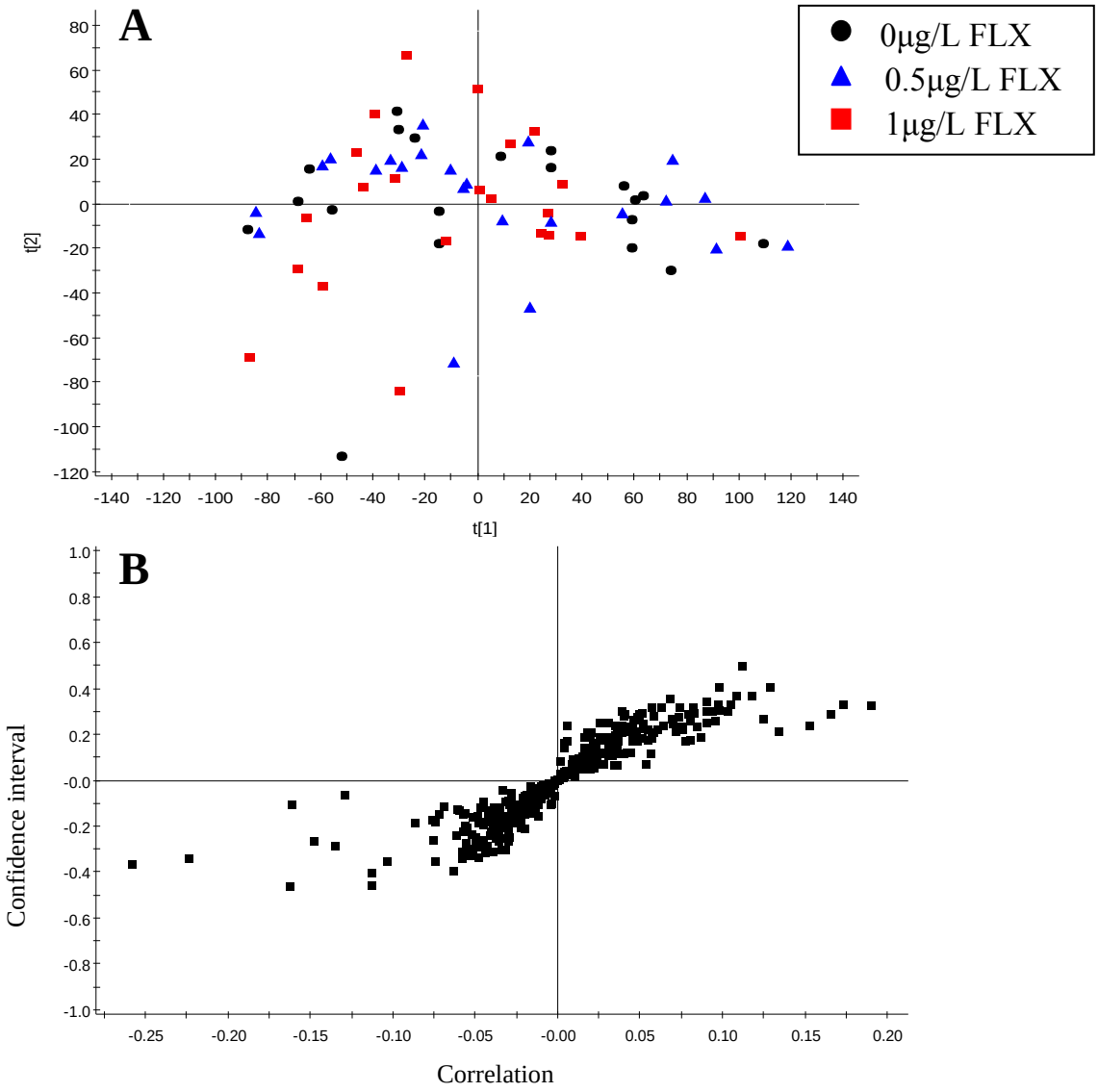

Figure 3.7. (A) PCA score plot of serum metabolite profiles of male and female goldfish from three fluoxetine (FLX) exposure groups (0, 0.5, and 1 µg/L) after a 7-day exposure and (B) OPLS-DA S-plot comparing the metabolites of 0 and 1 µg/L fluoxetine exposure groups. X-axis is principal component 1 (t[1]) and y-axis is principal component 2 (t[2]). For panel B, X-axis is correlation of metabolites between exposure groups and y-axis is confidence interval. Tissue samples were extracted and diluted with 75% acetonitrile and were analysed using UPLC-Q-TOF.

CHAPTER 4:

General Conclusions

4.1. Thesis results summary

Fluoxetine, the active ingredient in Prozac®, is found in the aquatic environment and causes metabolism disruption in exposed teleost fish. The objective of this research was to investigate the mechanisms involved in the metabolism disruption at environmentally relevant concentrations in the model goldfish. Two short-term waterborne fluoxetine exposures (7- and 14-day) were performed using two environmentally relevant doses of fluoxetine (0.5 and 1 μg/L) and goldfish tissues and biofluids (brain, liver, blood and bile) were analysed. From these samples, three potential indicators of metabolic disruption were considered: feeding neuropeptide mRNA levels, hepatic miRNA levels and metabolite profiles of blood and bile. By evaluating separate tissues and different endpoints, this series of experiments took a systems approach to assess the variable effects of fluoxetine on teleost fish metabolism.

4.1.1. Fluoxetine increases CRF mRNA in female goldfish brain

Initially, we hypothesized that fluoxetine acted at the brain to increase anorexigenic neuropeptide levels and reduce orexigenic neuropeptide levels to induce the well-known suppression of feeding response in fish[2,5,29]. Using RT-qPCR, we observed significant increases in female goldfish hypothalamus and telencephalon CRF mRNA following the 7-day fluoxetine exposure at 1 μg/L (Chapter 2). None of the other examined neuropeptide expression levels changed significantly in either male or female brain tissues (NPY, orexin-A, Sg-IIa, isotocin, CART-1, CCK). These findings support previous results of increased hypothalamic CRF mRNA in fluoxetine-exposed goldfish, which correspond with reduced NPY mRNA and decreased feeding in a previous study[5]. These results suggest that CRF may be one of the first feeding

neuropeptides to be affected by fluoxetine exposure and may cause a change in NPY mRNA and lowered appetite in goldfish[5].

4.1.2. Fluoxetine increases hepatic miRNA of goldfish

We also hypothesized that waterborne fluoxetine exposure may regulate hepatic miRNA abundance to modify metabolic activity in goldfish. In previous work, Craig et al.[6] used a microarray to identify six miRNAs in zebrafish liver that were increased following waterborne fluoxetine exposure. These miRNAs in zebrafish were predicted to regulate mRNA targets in the liver involved in insulin signalling, triacylglyceride synthesis and other metabolic pathways. Following our 7-day waterborne fluoxetine exposure, RT-qPCR revealed that five of the six hepatic miRNAs studied (*dre-miR-22b, dre-miR-140, dre-miR-210, dre-miR-301* and *dre-miR-457*) increased several fold in female goldfish liver at the highest dose (1 μg/L), while only one, *dre-miR-22b,* was increased in males (Chapter 2). After the 14-day exposure, *dre-miR-210, dre-miR-301, dre-miR-457* and *dre-let-7d* all increased by two fold in male livers at the highest dose. These findings suggest that though there are sex, dose, and time-dependant effects, fluoxetine may be acting at the liver to increase miRNA expression. While no known targets for these miRNAs exist in goldfish, previous studies have reported that miR-22 regulates components of the insulin pathway by targeting PI3K subunits in some fish species such as medaka, zebrafish, trout and stickleback[123]. In addition, *dre-let-7d* and *dre- miR-140* were predicted to negatively regulate the α1 and α2 mRNAs of AMPK in zebrafish, a key regulator of energy metabolism in animals[6]. Future work will involve predicting the mRNA targets in goldfish to compare the affected downstream hepatic pathways in goldfish. While preliminary, our results reinforce the concept that miRNA may be essential in regulating metabolic activity in the liver and are increased by fluoxetine exposure.

There is a possibility that the CRF mRNA increases in the hypothalamus and telencephalon and the hepatic miRNA increases following our fluoxetine exposure may be linked. Corticotropin-releasing factor was recently considered to play a role in the sympathetic regulation of hepatic pathophysiology in humans and CRF receptors, CRF_1 and CRF_2, were found in human liver [125]. Based on our results, future research should focus on the potential link between CRF and liver function by attempting to detect CRF or ACTH receptors in fish liver. The effects of fluoxetine on pituitary ACTH and interenal cortisol production should also be considered. This linkage between brain and liver may provide a new route through which fluoxetine disrupts metabolic processes.

4.1.3. Fluoxetine causes shifts in bile metabolite profiles of goldfish

Finally, we applied a discovery-driven metabolomic approach to explore potentially new metabolic effects caused by fluoxetine exposure. Following the 14-day and 7-day fluoxetine exposures, we investigated metabolite profiles of two biofluids, serum and bile, and proposed a null hypothesis that fluoxetine would not cause significant changes in either profile. Using multivariate analysis, no statistically significant metabolite changes were recorded across treatment groups in either tissue, thus we could not reject our initial null hypothesis (Chapter 3). This lack of observable effect was most likely due to the experimental set up and analytical techniques involved and therefore subtle differences in the serum and bile profiles of fluoxetine-exposed fish could not be properly recognized.

While the 7-day fluoxetine exposure was possibly too short to evoke observable metabolomic changes, the high exposure of fluoxetine (1 μg/L) in the 14-day exposure was likely to have caused differences in bile metabolite profiles. Principal component analysis of the 14-day exposure bile demonstrated that five out of the eight high exposure goldfish bile samples

separated clearly from the rest, which was confirmed statistically by nonparametric cluster analysis and canonical discriminate analysis. The lack of discriminant bile metabolites detected by OPLS-DA is presumably due to the inter- and intra-group variability of all of the other metabolites, which masked any subtle differences.

The serum of the high fluoxetine exposure group from the 14-day experiment also possibly contained discriminant metabolites that were not recognized by OPLS-DA. For example, one metabolite that was selected for identification despite not reaching statistical significance was tentatively identified as fluoxetine. This finding of fluoxetine in goldfish serum, if confirmed, verifies that fluoxetine was taken up by the waterborne exposure and re-affirms fluoxetine uptake in exposed fish tissues[30,128]. Other metabolites that were selected for cluster analysis may also therefore be worth tentatively identifying and may also contain useful phenotypic information regarding fluoxetine exposure.

Our results demonstrate that after 14 days, the higher exposure of fluoxetine caused metabolic changes in the bile, as observed by cluster analysis. Identifying the remaining of the metabolites used in cluster analysis may provide insight into altered phenotype of fluoxetine-exposed goldfish. In future metabolomic studies, more sensitive analytical tools are essential to reduce uncertainties and discrepancies in comprehensive data analysis of metabolite profiles.

4.2. Limitations

While the brain, liver and bile results demonstrate the effect of fluoxetine on feeding and metabolic disruption, there were several constraints in this study that limited our findings. In the neuropeptide studies, we did not consider peptide abundance and only measured levels of mRNA. There is a possibility that there is a difference between CRF mRNA abundance and CRF peptide levels, thus further studies to confirm true CRF peptide levels are important. In our hepatic

miRNA investigation, the number of samples used was relatively low compared to our neuropeptide mRNA data, reducing the power of our findings. Extraction of miRNA proved to be more difficult than mRNA extractions, which may have been the cause of the large error bars for the data set. To increase the impact of our hepatic miRNA findings in relation to phenotypic changes in the liver, future studies should attempt to predict the mRNA targets for the hepatic miRNA studied. Confirming the miRNA targets in goldfish would also be useful to complete our understanding of the relationship between fluoxetine, miRNA and metabolism disruption in goldfish more definitively.

Several changes should be made to the metabolomic assessment protocol to improve sample analysis and discriminant metabolite detection. To enhance the metabolite profile differences between exposure groups, higher concentrations of fluoxetine and longer durations of exposure should be used. Despite the decrease in environmental relevance, there would be a greater possibility of identifying differences between groups by exaggerating the exposure dose and duration. The metabolite profiles of tissues that are known to be fluoxetine targets, such as liver and brain, should also be assessed. While serum and bile are able to represent entire metabolome changes, the liver and brain tissues may be more demonstrative of fluoxetine changes due to the specific and unique action of fluoxetine on 5-HT transporters in these tissues. Several time points could also be taken to encompass the metabolic change experienced by individual fish and account for the variation within groups. Increased sample sizes would also increase the power of the statistical analyses. Sample preparation could be continuously improved to allow for a greater opportunity to include as many metabolites as possible, as the current protocol did not encompass the entirety of the serum or bile metabolomes. The variance

in future metabolomic data may be reduced if these modifications were adopted for future research.

4.3. Future directions

The challenge in environmental metabolomics is to confirm relationships between changes in metabolite profiles and changes in chemically-altered physiological endpoints, such as altered gene expression in the liver or brain. As such, future studies following this work should focus on connecting the metabolomic responses to fluoxetine exposure with physiological endpoints through phenotypic anchoring. Once several key candidates are identified by future metabolomic research, the potentially altered pathways should be investigated to help distinguish the toxicological effect from other changes unrelated to toxicity. By integrating the exploratory -omics methodology with other physiological and overt responses to a chemical, researchers can smooth the progress of interpreting multivariate metabolomics data[76]. Indeed, one environmental metabolomics review lists dozens of EDC metabolomic investigations that have been able to link metabolite profile changes to phenotypic responses to chemical exposure[77]. If a future study initially evaluated the brain and liver metabolomes of fluoxetine-exposed goldfish and identified potential biomarkers, the second phase of the study could link those markers to real brain and liver changes. This bottom-up and subsequent top-down technique will elicit greater confidence of the fluoxetine exposure effect on fish physiology.

4.4. General conclusions

In this series of experiments, we show that waterborne exposure to environmental levels of fluoxetine causes metabolic disruption at the level of the brain and liver, the effects of which are potentially identifiable in the bile in goldfish (see Fig. 4.1 for a generalized summary). These results support and extend the idea that fluoxetine acts on fish at multiple organ levels involved

with feeding[2,5,29], stress[41,42,44], metabolism[6] and excretion[128]. This research therefore indicates that an environmental dose of a common SSRI may be impacting multiple tissues and multiple systems in addition to the intended targets in the brain. Due to the widespread detection of fluoxetine and other SSRIs in the aquatic environment, future research should continue to explore the mechanisms through which these EDCs disrupt multiple aspects of fish physiology.

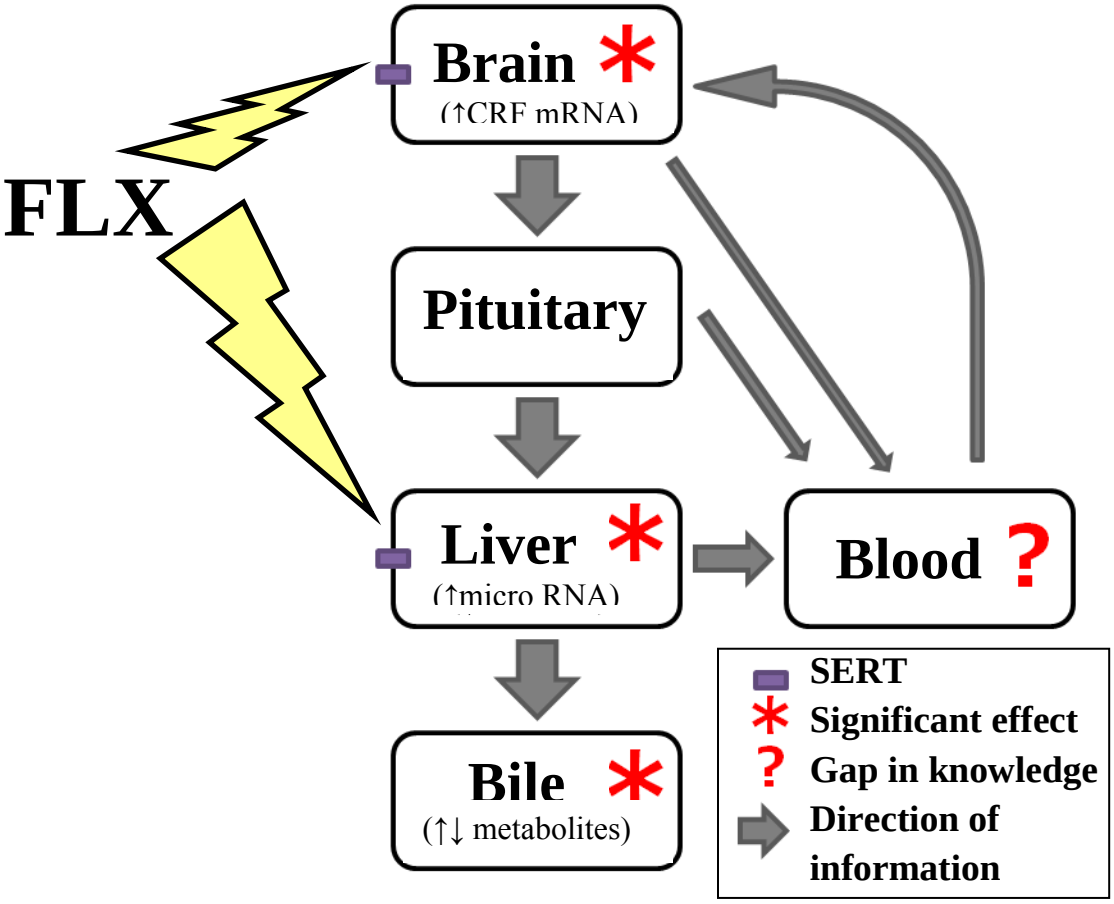

Figure 4.1. A summary of the observed effects of fluoxetine (FLX) on feeding and metabolism in goldfish, with the known locations of the fluoxetine target, the serotonin reuptake transporter (SERT), and the flow of information regarding metabolic activity indicated. 7-day fluoxetine exposure at 1 µg/L resulted in corticotropin-releasing factor (CRF) mRNA levels to significantly increase in female hypothalamus and telencephalon (Chapter 2) in the brain, *dre-let-7d*, *dre-miR-22b*, *dre-miR-140*, *dre-miR-210*, *dre-miR-301a* and *dre-miR-457b* to increase in the liver (Chapter 2), and resulted in a significant shift in the bile metabolite profiles of goldfish exposed to 1 µg/L fluoxetine for 14 days (Chapter 3).

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