CHARACTERIZING TOXICITY PATHWAYS OF FLUOXETINE TO PREDICT ADVERSE OUTCOMES IN ADULT FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

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ABSTRACT

Current ecotoxicity testing programs mandated by regulatory agencies are impeded as they predominantly rely on slow and expensive animal tests measuring traditional adverse outcomes such as mortality, growth, disease, reproductive failure, or developmental dysfunction. To address these concerns and support environmental risk assessment, the development of new approach methodologies (NAMs) is increasingly involving short-term mechanistic assays that employ molecular endpoints, such as transcriptomics and proteomics, to predict adverse outcomes of regulatory relevance. The research in this thesis aimed to use fluoxetine (FLX) as the model compound for the development of a novel mechanism-based toxicity assay through elucidation of its molecular toxicity pathways in adult fathead minnows. Specifically, the objectives of this study were to characterize the relationships between molecular response patterns using whole proteomics and transcriptomics and apical level effects of regulatory relevance (fecundity and histopathology). In two parallel studies, fish were exposed to three FLX concentrations (measured: 2.42, 10.7, and 56.7 μgL^{-1}) and a control. After the 96-hour exposure, molecular response signatures were characterized using whole proteomics and transcriptomics analyses in livers and brains of exposed male fish. Following the 21-day exposure, fish were sampled and assessed for liver histopathology and morphometric measurements. Fecundity was monitored throughout the study and revealed a significant reduction at all FLX-treatment levels. Hepatic histopathological assessment found presence of lipid-type vacuolation in two of five specimens of fish exposed to 56.7 μgL^{-1} FLX. Whole transcriptomic analysis in the liver revealed dysregulation of pathways associated with biosynthesis and metabolism of fatty acids, which may be an upstream molecular response that led to lipid-type vacuolation of hepatocytes, as observed in the histology analysis. Whole proteome analysis of the same fish revealed dysregulation of several processes including PPAR signalling.

These molecular signatures may be upstream responses that led to lipid-type vacuolation of hepatocytes. Upregulated genes in the brain suggested alterations in serotonin-related signalling processes and reproductive behaviour, which may explain the observed significant decrease in fecundity. While the relationships between molecular responses and adverse outcomes remain complex, this research provided important insights into the mechanistic toxicity of FLX. This work achieved the research objectives in demonstrating the potential of large-scale omics data to elucidate the complex physiological response of adult fathead minnows to FLX as well as added to the growing body of literature on the utility of these methods in support of chemical hazard assessment.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
$\mu g g^{-1}$	micrograms per gram
$\mu g L^{-1}$	micrograms per litre
µM	micromolar
µm	micrometres
5-HIAA	5-hydroxyindole-3-acetic acid
5-HT	serotonin; 5-hydroxytryptamine
AChE	acetylcholinesterase
ANOVA	analysis of variance
AO	adverse outcome
AOP	adverse outcome pathway
AVT/AVP	arginine vasotocin/vasopressin
BH	Benjamini-Hochberg
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
cm	centimetres
d5-FLX	deuterated fluoxetine
DAP	differentially abundant protein
DEG	differentially expressed gene
DNA	deoxyribonucleic acid
FASP	filter-aided sample preparation
FDR	false discovery rate

FHM	fathead minnow
FLX	fluoxetine
g	grams
GABA	gamma-aminobutyric acid
gL^{-1}	grams per litre
GO	gene ontology
HDL	high-density lipoprotein
hpf	hours post fertilization
HPG	hypothalamic-pituitary-gonadal
HPLC	high-performance liquid chromatography
HSI	hepatosomatic index
IP	intraperitoneal
K	condition factor
KE	key event
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	litre
Lkg^{-1}	litres per kilogram
logKow	octanol-water partition coefficient
MIE	molecular initiating event
miRNA	micro ribonucleic acid
mm	millimetres
mRNA	messenger ribonucleic acid
MS	mass spectrometry

NAMs	new approach methodologies
nFLX	norfluoxetine
ngL^{-1}	nanograms per litre
ngmL^{-1}	nanograms per millilitre
PAFs	platelet-activating factors
PCR	polymerase chain reaction
PPAR	Peroxisome proliferator-activated receptor
PPCPs	pharmaceuticals and personal care products
qPCR	quantitative polymerase chain reaction
QQ	quantile-quantile
qRT-PCR	quantitative real-time polymerase chain
REACH	Registration, Evaluation, Authorization, and Restriction of Chemicals
RIN	RNA Integrity Number
RNAseq	ribonucleic acid sequencing
SPE	solvent phase extraction
SSRI	selective serotonin reuptake inhibitor
UTR	untranslated region
v/v	volume per volume

PREFACE

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate Studies and Postdoctoral Research guidelines for a manuscript-style thesis. Therefore, there is some repetition between the content presented in each chapter. Chapter 1 is a general introduction and literature review, including project goals and objectives. Chapter 2 is organized as a manuscript for publication in a peer-reviewed scientific journal and a description of author contributions is provided in the preface of this chapter. Supplementary materials that will be published from Chapter 2 has been included in the Appendices section at the end of this thesis. Chapter 3 is a general discussion and overall conclusion. References cited in each chapter are combined and listed in the References section of this thesis.

CHAPTER 1: INTRODUCTION

1.1 Predictive Ecotoxicology & Adverse Outcome Pathways

A critical challenge in environmental risk assessment is the large number of chemical substances that currently require toxicity testing as well as the continuous growth of the number of contaminants entering the environment each year. In recent years, there has been an increasing societal awareness and concern regarding the great number of chemical contaminants for which only limited hazard information is available. Legislators in Europe and North America are taking responsibility for this issue by implementing large-scale efforts such as the REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) program in Europe that was created to identify, prioritize, and take action on harmful chemicals (Petry et al., 2006). The demand for toxicity testing as a result from the REACH program is estimated to use 54 million vertebrate animals and cost US $13.6 billion (Basu et al., 2019; Hartung and Rovida, 2009).

Current ecotoxicity testing programs performed by regulatory agencies are conducted using predominantly *in vivo* tests measuring traditional adverse outcomes such as mortality, growth, disease, reproductive failure, or developmental dysfunction (Villeneuve and Garcia-Reyero, 2011). However, researchers in the field of ecotoxicology are increasingly applying new approach methodologies and next generation technologies including *in vitro* studies, whole transcriptomics, proteomics, metabolomics, and high-throughput technologies. These methods enable risk assessors to examine the mechanisms of toxic effects at the cellular level of an organism as well as to improve efficiency, economy, and reliability of data collection (Basu et al., 2019; Villeneuve and Garcia-Reyero, 2011). Additionally, gene expression profiles can be used to compare differences and similarities of toxic responses across different organisms. This can be useful in environmental risk assessment by maximizing the use of existing biological and

toxicological knowledge to be applied to non-model species. By effectively predicting the most likely sensitive endpoint of a toxicant, the need to conduct a suite of tests with live animals could potentially be significantly reduced, and therefore save time, resources, and animals.

A new strategy that has been proposed to address some of the current issues with chemical safety assessments is the adverse outcome pathway (AOP) framework that has gained increasing attention over the past decade. An AOP is a systematic approach for describing the scientifically credible basis for linking a toxicant-induced molecular initiating event (MIE) to an adverse outcome (AO) through a series of key events (KEs) across biological levels of organization (Ankley et al., 2010; Ankley and Edwards, 2018). The AOP framework could play an important role in the application of predictive toxicology techniques to chemical risk assessment by translating molecular mechanistic toxicity data generated through novel 'omics techniques (e.g. identification of molecular toxicity patterns obtained through short term, early life-stage or *in vitro* studies) to regulatory decision-making (Hecker and LaLone, 2019; LaLone et al., 2017). However, AOPs are somewhat limited in that they currently only provide a qualitative description of the relationship between the MIE, KEs, and the AO. Thus, there is need for more quantitative approaches. Recent efforts have focused on the identification, development, and evaluation of new AOPs based on 'omics, high-throughput data, system modelling, and repositories of curated toxicity information (Ankley and Edwards, 2018). Specifically, investigations of 'omics responses are useful in the development of AOPs by screening molecular signals and identifying and linking MIEs to KEs (Huang et al., 2017).

1.2 Toxicogenomics

The study of toxicogenomics is the investigation of the relationship between structure and activity of the genome and the resulting adverse effects (Aardema and MacGregor, 2002). The key

advantage of using omics methods in a systems approach is the opportunity to simultaneously assess big data sets of molecular responses, facilitating a more comprehensive understanding of the molecular processes that drive an organism's physiological status. There are three primary technologies that make up and contribute to toxicogenomics: transcriptomics, proteomics, and metabolomics. These three technologies utilize global or targeted methods in measuring families of cellular molecules (mRNA, protein, and metabolites, respectively).

Transcriptomics include technologies such as quantitative PCR (qPCR) and microarrays; however, the most advanced technology is RNA sequencing (RNAseq). Whole transcriptome sequencing using RNAseq allows for examination of the entire suite of genes expressed by an organism in a high-throughput and quantitative manner with high resolution, and therefore, is sensitive to detecting novel transcripts (Wang et al., 2009). RNAseq technology works by estimating the expression level of a transcript from the number of reads that map to that transcript. The read count of a given gene is related to both its expression level and length of the transcript. This can result in the introduction of selection bias because longer or highly expressed transcripts are more likely to be detected for differential expression between transcripts and therefore, requires normalization (Young et al., 2010).

Gene Ontology (GO), often used in interpretation of differential expression of transcriptome, is a collaborative project that provides a structured and controlled vocabulary and classification of biological domains that is continuously updated by expert curators (Mlecnik et al., 2018). A GO analysis can be advantageous to a researcher because it allows for focus on a functional data set and account for biological variation (Simmons et al., 2015). Variability in the transcriptome is naturally occurring and can lead to tolerance of environmental stressors. Some networks of molecular responses are typically more stable than others; for example, transcripts

involved in transcription and translation may be expected to be more stable than those involved in metabolic processes (Martyniuk and Houlahan, 2013). Genes with lower natural variability may be more informative bioindicators of effect, as perturbations due to xenobiotic exposure can be interpreted as a serious problem. Investigations in the variability of the transcriptome are useful because changes in gene expression due to toxicity can be separated out from the noise of natural variability.

Proteomics is the analysis of the function and structure of proteins in a tissue sample and their relative abundance or modification (Aardema and MacGregor, 2002; Villeneuve and Garcia-Reyero, 2011). Essentially, whole proteomics techniques interrogate the entire repertoire of proteins of a given sample and can identify interactions of proteins involved in diverse cellular function (Mlecnik et al., 2018). While theoretically translation of mRNA into proteins should be proportional to gene transcript levels, dysregulation seen in the transcriptome may not be directly correlated with alterations of the proteome because the amount of a particular protein also relies on synthesis and degradation pathways as well as post-transcriptional changes. Therefore, combining transcriptomics and proteomics technologies will allow for a more reliable discovery of biological and cellular functions, mechanisms of action of potentially toxic chemicals, and predictive biomarkers of effect (Mlecnik et al., 2018). Often, the proteome is considered to be more complex than the genome because of factors such as post-translational modifications, differences in the functional proteome among cell types, and varying isoforms of a given protein (Garcia-Reyero and Perkins, 2011). Metabolomics technology is the global analysis of endogenous small molecule metabolites and their relative abundance in a tissue sample, and therefore is regarded as a comprehensive evaluation of biological responses/activity at a given time (Huang et al., 2016; Villeneuve and Garcia-Reyero, 2011).

Inclusion of multi-omics analyses in studies investigating the mechanisms of toxic action of xenobiotics provides the opportunity for the improved characterization of the underlying molecular causes leading to a biological response, and therefore enhances prediction of toxicological outcomes in whole animals in the absence of morphological effects (Huang et al., 2017). However, responses at the transcriptome, proteome, and metabolome levels do not necessarily occur concurrently and cannot be assumed to be permanently affected (Simmons et al., 2015). This is particularly true if the observed responses are compensatory or adaptive. Therefore, it is important to consider the timing of sampling in an exposure trial.

1.3 Fathead Minnow as a Model Lab Species

The fathead minnow (*Pimephales promelas*) has been used extensively in toxicity testing and is considered an ideal model lab species since the 1950s (Ankley and Villeneuve, 2006). Fathead minnows are a small-bodied, warm water fish that are native to freshwater lakes and rivers across North America and are therefore an environmentally relevant species to use in toxicological studies. The species has well-defined and described developmental life stages from fertilization and hatch to sexual maturation at four to five months of age. For this reason, fathead minnows are often used to assess the effects of contaminants on reproductive endpoints such as vitellogenin, secondary sex characteristics (i.e. dark banding, dorsal pad, and nuptial tubercles on males), and quantitative fecundity (Ankley and Villeneuve, 2006; OECD, 2012). The species has been used to characterize the toxicity of a vast number of chemicals in thousands of tests ranging from acute to sub-chronic to chronic exposures (Ankley and Villeneuve, 2006). Toxicity tests with fish species are important to further our understanding of the environmental impacts of chemicals; however, it is not always feasible to use native species that are threatened or not easily held captive. The

fathead minnow is a popular test organism because it is relatively easy to maintain in a culture and are fairly tolerant to various water types and conditions (Ankley and Villeneuve, 2006).

The evolving field of toxicogenomics research has previously been limited regarding the fathead minnow due to the lack of availability of a fully annotated genome. To address this, a recent and important step by Burns et al. (2016) was the sequencing and assembly of the fathead minnow genome. However, these assemblies were unannotated and without indication functional components. Recent work has produced annotations of the fathead minnow genome were developed by Saari et al. (2017) and Martinson et al. (n.d.), which can expedite a wide range of molecular analyses and applications with this species. For example, it can aid in the development of PCR primers, providing a more complete and unbiased view of the transcriptome to allow for identification of novel transcripts, cross-validation of the proteome, detection of different gene isoforms, and the comparison of genes between fathead minnows and other species (Saari et al., 2017). In-house efforts have been implemented by our research group to functionally annotate the latest fathead minnow genome to be applied to our greater effort of identifying key molecular signatures that can be predictive of adverse outcomes of regulatory relevance. These applications will continue to benefit the use of the fathead minnow as a lab model species.

1.4 PCPPs in the Aquatic Environment

The occurrence of pharmaceuticals and personal care products (PPCPs) in the aquatic environment has become increasingly widespread, especially in waters receiving municipal wastewater and sewage treatment effluent. Because of their persistent input and constant presence, PPCPs have become a toxicological threat to both human and ecological health (Ebele et al., 2017). Concentrations of PPCPs found in the environment are typically low ($<\mu gL^{-1}$); however, PPCPs are intended to be bioactive at low concentrations, and therefore have the capacity to elicit toxic

effects at levels below those of other contaminants (Huang et al., 2017). Aquatic species, including fish, inhabiting effluent-receiving waters are at a greater risk of exposure and adversities resulting from toxicity of PPCPs. Due to its wide use and persistence in surface waters, fluoxetine (FLX) is considered a PPCP of particular concern for environmental contamination.

1.5 Fluoxetine: Contaminant of Concern

1.5.1 Usage & Mechanism of Action

FLX, the active ingredient in Prozac® (Eli Lilly), is a selective serotonin reuptake inhibitor (SSRI) often prescribed to treat depression, bulimia nervosa, and obsessive-compulsive disorder. Prozac® was initially approved by the U.S. Food and Drug Administration in 1987 and has since had a steady increase in prescriptions across the United States and Canada. During 2011 – 2014, 12.7% of persons over the age of 12 in the United States reported the use of antidepressants (Pratt et al., 2017). FLX is one of the most widely prescribed antidepressants, making up a large constituent of this group with over 24 million prescriptions filled in the United States alone in 2010 (Wenthur et al., 2014).

In order to reach and maintain their desired therapeutic effect, SSRIs require several months of chronic administration to the patient (Kreke and Dietrich, 2008). FLX produces its antidepressant effect by reducing the clearance rate of serotonin (5-hydroxytryptamine; 5-HT) through binding to pre-synaptic serotonin transporter; therefore, potentiating the action of 5-HT. 5-HT can also act by "volume transmission," meaning that it can be released outside the synapse and exert systemic effects on surrounding cells. Furthermore, chronic use of the SSRI will lead to adaptive changes in neuronal function through the desensitization of the 5-HT receptors (NCBI, 2018). Serotonin is a monoamine neurotransmitter synthesized from the essential amino acid tryptophan within enterochromaffin cells of the gastrointestinal tract (O'Mahony et al., 2015). The

neurotransmitter is found in a variety of tissues and cells including myenteric cells in the digestive system, beta-cells in the pancreas, parafollicular cells in the thyroid, dorsal root ganglia, and taste buds (Lillesaar, 2011). Moreover, serotonin is also the precursor for the neurohormone melatonin which is synthesized within the pineal gland. 5-HT has been shown to regulate cellular functions including cell proliferation, neuronal differentiation, neurite growth, and synaptogenesis (Lillesaar, 2011). Decades of research has clarified that the serotonergic system is involved in a variety of functions in vertebrates such as sensation, memory, behaviour, wakefulness, aggression, and appetite (Müller and Homberg, 2015; Winder et al., 2009). It is now widely accepted that 5-HT is involved in many, if not all, other transmitter and signalling systems (Müller and Homberg, 2015). The brain-gut axis functions with 5-HT as a key signalling molecule in both the enteric nervous system and the central nervous system (O'Mahony et al., 2015). Rhythmic locomotor movements is influenced by the serotonergic system by innervating motor neurons projecting to axial muscles (Lillesaar, 2011).

Following oral administration, FLX is well absorbed from the gastrointestinal tract with an oral bioavailability of 60-80% (NCBI, 2018). FLX binds with high affinity to plasma proteins, with albumin and α-1-acid glycoprotein as its principal plasma binding proteins (DeVane, 1999). The volume of distribution of fluoxetine is 20-45 L kg^{-1} (NCBI, 2018). In humans, FLX is metabolized in the liver via *N*-demethylation pathway with an isoenzyme of cytochrome P450, CYP2D6, to form the principal metabolite norfluoxetine (nFLX), which is equally bioactive as FLX (Asensi-Bernardi et al., 2013; Kreke and Dietrich, 2008; Silva et al., 2012). Both the parent compound and the metabolite are known to also undergo phase II glucuronidation reactions in the liver (NCBI, 2018). It has been reported that 11% of administered FLX is eliminated unchanged by the kidneys while 7% is eliminated as nFLX (Mennigen et al., 2010b). Furthermore,

approximately 80% of FLX is excreted as the parent compound, nFLX, or glucuronides of either compound (Wenthur et al., 2014).

The 5-HT transporter and receptors are known to be evolutionarily and functionally conserved across the animal kingdom, and therefore, FLX can also elicit its effects on nontarget species, including fish. (McDonald, 2017). There have been two subtypes of the 5-HT transporter identified in zebrafish as opposed to only one in mammals (Amador and McDonald, 2018). The affinity of the transporters is lower in fish than mammals, although the efficacy of SSRI inhibition is similar. Teleosts have 13 unique subtypes within seven families of the 5-HT receptor with varying function and affinity depending on the tissue type, which could result in a variety of physiological responses. Therefore, this complicates the read-across hypothesis, an assumption that the compound will elicit the same pharmacological effect in a non-target organism if the dose and molecular target is conserved. Of the 13 subtypes, all are G-protein coupled receptors apart from 5-HT_3, which is a cation channel. Activation of the five subtypes of the 5-HT_1 family and 5-HT_{5A} will result in the inhibition of adenylyl cyclase activity; conversely, 5-$HT_{4,6,7}$ will result in activation of adenylyl cyclase. Additionally, when present on the presynaptic membrane, the 5-HT_1 family will act as auto-receptors by reducing synthesis and release of 5-HT from the presynaptic cell. The subtypes of the second receptor family result in the activation of phospholipase C. McDonald (2017) suggests that the diversity of 5-HT receptors provide a organism with plasticity in response to physiological and environmental challenge. When exposed to high doses of SSRIs, a systemic change in extracellular 5-HT may occur; this, in turn, could impact varying types of 5-HT receptors throughout the body. Furthermore, this could explain atypical dose-responses to SSRIs.

1.5.2 Source, Fate, & Occurrence in the Aquatic Environment

The introduction of pharmaceuticals, such as FLX, into surface water is attributed, in part, to the quantity of the manufactured drug, the dosage frequency and amount, and disposal of unused/expired drugs through municipal sewage systems and leaching from landfills (Daughton and Ternes, 1999). In addition, during wastewater treatment, the tendency for pharmaceuticals to either sorb to filterable solids or undergo microbial degradation to lower molecular weight products will influence whether, or to what extent, they will enter effluent-receiving waters (Daughton and Ternes, 1999). Increased environmental prevalence of pharmaceuticals, such as FLX, could also vary depending on region, time of year, and the wastewater treatment level of a municipal wastewater treatment plant. For example, seasonal variation can be attributed to the observation that FLX and other SSRI prescriptions may peak during fall and winter due to a higher incidence of anxiety and depression (Kreke and Dietrich, 2008). Considering that the FLX prescription rate has been increasing over recent decades and the per capita wastewater effluent to surface water ratio steadily increases, it can be expected that the environmental concentrations of pharmaceuticals such as FLX will also increase (Kreke and Dietrich, 2008; Pratt et al., 2017). Additionally, other possible sources of contamination include leaching from landfills and pharmaceutical manufacturing plants (Schultz et al., 2011).

The median concentration of FLX that has been observed in surface waters is 17.8 ngL^{-1}; however, upwards of 596 ngL^{-1} have been reported in some cases (Hughes et al., 2013). The concentrations observed in wastewater effluent can vary by municipality: for example, Schultz and Furlong (2008) observed 58 ngL^{-1} in wastewater effluent from a metropolitan urban centre in 2006 in Texas and up to 20 ngL^{-1} in downstream surface waters. In California, wastewater effluent from two treatment plants reportedly contained 0.01 μgL^{-1} and 0.04 μgL^{-1} (Vidal-Dorsch et al., 2013).

In 2002, Metcalfe et al. (2003) measured between 38 – 99 ngL^{-1} in various wastewater effluents of cities on the Great Lakes in Canada. From 1999 – 2000, 84 streams were sampled across the US by Kolpin et al. (2002) and analyzed for FLX; they found an average concentration of 12 ngL^{-1}.

The concentrations typically recorded in the aquatic environment are sub-therapeutic, and are therefore negligible with regard to their acute toxicological risk (Bringolf et al., 2010). The photolysis half-life of FLX in the environment has been reported to be 122 days (Black and Armbrust, 2007). In addition, the biological half-life is between 4 – 6 days for the parent compound and 4 – 16 days for the potent metabolite nFLX (NCBI, 2018). The octanol:water partition coefficient, $logK_{ow}$, a measure of lipophilicity and used for predicting uptake of xenobiotics, of FLX has been reported at contradicting levels between 1.22 – 3.93, but the chemical is generally considered to be relatively soluble in water (Black and Armbrust, 2007; Brooks, 2014). Due to its constant introduction into aquatic environments and hydrolytic and photolytic stability, FLX is considered to be a pseudo-persistent contaminant, and therefore has the potential to elicit chronic effects (Kwon and Armbrust, 2006).

Pseudo-persistent scenarios are defined when the chemical half-life is exceeded by the effluent introduction rate; therefore, organisms are continuously exposed (Schultz et al., 2011). Similarly, a true persistent chemical has an innate ability to resist degradation, and therefore has the potential for bioaccumulation and long-range transport (Boethling et al., 2009). Like a persistent pollutant, FLX can bioaccumulate in aquatic organisms such as fish. A study by Brooks et al. (2005) provided the first indication that SSRIs and their metabolites may accumulate in fish residing in effluent-dominated waterways. Analyzing the brain, liver, and muscle tissues of three native fish species in a stream in Texas, they found that FLX accumulated most in the brain followed by the liver and muscle with concentrations of 1.58, 1.34, and 0.11 ng/g, respectively

(Brooks et al., 2005). Their results also showed greater concentrations of nFLX compared to FLX in all tissues suggesting slower accumulation of the parent compound.

1.6 Toxicity of Fluoxetine in Fish

1.6.1 The Read-Across Hypothesis

Rand-Weaver et al. (2013) discuss the "Read-Across Hypothesis", which suggests that if a molecular target is conserved across species including non-target organisms, such as fish in effluent-receiving waters, tissue concentrations of a pharmaceutical similar to human therapeutic levels will have a comparable pharmacological effect, including side effects. When molecular targets of pharmaceuticals are conserved, utilizing read-across can be useful in environmental risk assessment because, unlike traditional approaches, it can apply the therapeutic mechanism of action to predict potential environmental impacts (Berninger and Brooks, 2010).

In the case of FLX, there is supporting evidence that read-across can be used as an approach to support environmental risk assessment for this compound. The 5-HT receptor, and molecular target of FLX, has been found to be conserved across vertebrates (Beulig and Fowler, 2008; Cunha et al., 2018; Kreke and Dietrich, 2008). Several studies demonstrated that the effects of FLX observed in fish were somewhat predictable from human therapeutic effects. For example, Margiotta-Casaluci et al. (2014) observed an anxiolytic behavioural response in adult fathead minnows exposed to an aqueous exposure of 72 μgL^{-1}, which resulted in a plasma concentration that fell within the range of the human therapeutic level. Because no anxiolytic effects were observed at plasma concentrations below the human therapeutic plasma concentration range, the authors concluded that this study validates the read-across hypothesis, and that a cross-species extrapolation approach can be applied to assess sensitivity of fish to pharmaceuticals.

Contrary, there is also an argument against the read-across hypothesis to be made. Some studies have shown that the read-across hypothesis does not necessarily predict effect concentrations in fish because impacts of FLX and other SSRIs have been produced at steady state plasma concentrations well below the human therapeutic plasma concentration (Henry and Black, 2008; Pelli and Connaughton, 2015). There are key pharmacokinetic properties, such as absorption rate, half-life, and metabolism, that are highly variable between humans and fish that may affect how FLX produces its response (McDonald, 2017). The primary enzyme responsible for the metabolism of FLX, CYP2D6, is not expressed in some fish species such as *Pimephales promelas* (Margiotta-Casaluci et al., 2014; McRobb et al., 2014). Because fish are poikilotherms, they have inherently lower metabolic rates than mammals. Thus, fish may have slower uptake and clearance rates for compounds like FLX. Therefore, the toxicokinetics of FLX in fish are not necessarily comparable to those in humans and mammals.

The affinity of 5-HT to the transporter is much higher in mammals than in fish, and therefore, they are much more sensitive (Amador and McDonald, 2018). When comparing fish to mammals, there is only about a 60-70% similarity in sequence identity among human toxicity targets (McRobb et al., 2014). Specifically, there is about 65% similarity between the transcript sequence of the 5-HT transporter between fish and mammals (Amador and McDonald, 2018). This indicates that effects cannot be directly extrapolated. Consequently, when researching environmental impacts of pharmaceuticals such as FLX, the read-across hypothesis can be considered as a starting point but should not be used as risk assessment tool.

1.6.2 Adverse Outcome Effects

There is current evidence that shows that some effects of FLX in fish do not align with the "Read-Across Hypothesis" but rather exceed expectations of anxiolytic behavioural outcomes.

For example, some studies have observed that FLX exposure can affect male fish mating behaviour in addition to increased aggression, isolation, and repetitive behaviour and decreased predator avoidance behaviour (Beulig and Fowler, 2008; Painter et al., 2009; Winder et al., 2009). Theodoridi et al. (2017) conducted a study with male wildtype zebrafish to determine changes in social interactions. The experiment consisted of two males with a strongly established dominant and subordinate relationship exposed to 5 mgL^{-1} FLX, based on previously published research, for a duration of 2 hours (n=8 pairs). Behaviour was observed using slow-motion video and compared to the control groups; they found significantly fewer attacks on each other, and the dominant fish spent less time chasing the subordinate. Acute immersion in a high dose of FLX caused a significant decrease in the aggressive behaviour of the dominant fish and increased the confidence of the subordinates. Additionally, expression of selected genes involved in the serotonergic system were analyzed using qPCR, and it was discovered that, generally, the dominant zebrafish had more differentially regulated genes than the subordinate (see section 1.6.3). The authors hypothesized that certain dysregulated genes may be involved in attenuation of defensive aggression. Therefore, fluoxetine exposure may be changing the social dynamics in male zebrafish.

A well-studied escape response, the c-start, is a reflex behaviour conserved across teleost species as a predator avoidance strategy. Therefore, this behaviour can be used in studies to analyze the effects of FLX on predator avoidance. Such a study was performed by Painter et al. (2009) in which they analyzed larval fathead minnows exposed to a range of environmentally relevant concentrations of FLX (0, 25, 125, and 250 ngL^{-1}) until 12 days post-hatch. By recording with a high-speed camera, they could observe the larvae's c-start response when provoked by a vibration to simulate a predator. The study revealed that there was a significantly slower escape velocity in a dose-dependent relationship as compared to the control group. This response could

have serious implications in the species survivability in the environment as it is an indication of reduced survivability and reproductive fitness.

The effects of FLX on the reproductive success of fish have been somewhat limited in the current literature. In teleost fish, the hypothalamic-pituitary-gonadal (HPG) axis is responsible for the regulation of reproduction (McDonald et al., 2011). At all levels, 5-HT will regulate the HPG axis by stimulating the release of important hormones involved in reproduction (Mennigen et al., 2008). Disruption of the HPG axis by the exposure of SSRIs in male fish has been shown to cause a reduction in spermatogenesis, delay the development of secondary sex characteristics, decrease milt volume, and induce vitellogenin production (Henry and Black, 2008; Mennigen et al., 2010a; Prasad et al., 2015; Schultz et al., 2011). A study by Lister et al. (2009) demonstrated that sexually mature zebrafish exposed to 32 μgL^{-1} FLX spawned significantly fewer eggs than the control group. Few other studies have analyzed the effects of FLX on the fecundity of fish species. In contrast, a study with Japanese medaka exposed to environmentally relevant concentrations of FLX for four weeks yielded no significant change in egg production, rate of fertilization, and rate of spawning (Foran et al., 2004). Sebire et al. (2015) examined the effects of FLX on reproductive endpoints in three-spined sticklebacks following a 21-day exposure. Change in embryos spawning was not measured directly, but the authors did observe a significant decrease in nest quality, a critical factor in stickleback reproduction, in groups exposed to aqueous FLX at comparable concentrations, indicating a challenge in attracting female partners (Sebire et al., 2015).

An investigation by Weinberger II and Klaper (2014) involving adult fathead minnows assessed changes in mating behaviour when exposed to a range of FLX concentrations (0, 0.1, 1, 10, & 100 μgL^{-1}) chronically for 4 weeks. Their research examined several reproductive parameters: quantification of male visits to breeding tiles, females swimming under the tiles, male

attacks on females, males chasing females, and nest preparation and maintenance. FLX concentrations as low as 1 μgL^{-1} were reported to impact male nest preparation, maintenance, and defence. Interestingly, they found that at 100 μgL^{-1} the male fish had an increase in aggressive behaviour and reported this to correlate with a decrease in female survivorship (33%.) No change in egg production was observed; however, they hypothesized that the effects of male nest defence could potentially have an adverse impact on offspring survivorship in the environment.

Schultz et al. (2011) conducted a 21-day exposure of 2.5 and 28 ngL^{-1} FLX to male fathead minnows. The objectives of the study were to characterize whether FLX uptake is selective and if it affects reproductive anatomy, physiology, and behaviour. In both dosed treatment groups, the concentrations of FLX in brain tissue were considerably higher than suggested by the concentration in the water. This indicates that FLX may have a great potential for bioaccumulation in the central nervous system. Other notable effects of FLX at 28 ngL^{-1} included a significant induction of vitellogenin production, hypertrophy of testicular tissue, and decrease in secondary sex characteristics such as nuptial tubercles, dorsal fat pad, and colouration. These effects indicate that there is disruption of the endocrine and reproductive systems of male fathead minnows when exposed to environmentally relevant concentrations of FLX.

Morando et al. (2009) examined the potential sensitivity of branchial nitrogen excretion and intestinal osmoregulation of gulf toadfish exposed to FLX by intraperitoneal injection. The concentrations tested were 25, 50, 75, and 100 $\mu g\ g^{-1}$ FLX dissolved in coconut oil. The fish with the two highest doses had the lowest survivalship (<25%, n=16), and were not used for further analyses. The lowest dose, 25 μgg^{-1}, of the exposure caused an increase in urea and ammonia excretion compared to the initial levels, which was measured in tank water samples. The fish exposed to 50 μgg^{-1} FLX had 3-fold greater concentrations of circulating cortisol than the control

group. At doses of 25 and 50 $\mu g g^{-1}$ there was a significant decrease in osmolality and increase in blood 5-HT. An increase in 5-HT in the blood could have vital physiological implications because the 5-HT receptor is present on blood platelets, lymphocytes, and gastrointestinal enterocytes (Lesch et al., 1993; Lima and Urbina, 2002; Martel, 2006). Therefore, the consequences of FLX exposure could be widespread, and physiological processes that are sensitive to 5-HT may be particularly susceptible.

As reviewed by McDonald (2017), 5-HT plays an important role in the stimulation of smooth muscle contractions of the gastrointestinal tract in a variety of fish species. Interestingly, the intestines contain a high expression of 5-HT, and therefore could respond to SSRI exposure. Furthermore, the enterochromaffin cells and enteric neurons of the intestinal epithelial layer stores 95% of 5-HT within a vertebrate body (Njagi et al., 2010). Teleost species, such as fish of the family Cyprinidae, tend to have a low density of mucosal enterochromaffin cells and a high abundance of 5-HT-rich enteric neurons (Velarde et al., 2010). However, few studies have investigated the potential impacts of SSRI exposure on the gastrointestinal tract. Evidence from Morando et al. (2009) indicates that toadfish experience stimulation of intestinal fluid absorption and disruption in plasma osmolality after 24 hours of a treatment of 25 $\mu g g^{-1}$ FLX. Further research needs to be conducted to determine the implications of FLX on the gastrointestinal tract in fish.

Gaworecki and Klaine (2008) conducted a study to determine the effects of waterborne FLX on the feeding behaviour of hybrid striped bass (*Morone saxatilis* × *M. chrysops*) and the association to changes in brain serotonin levels. The concentrations tested (0, 35, 75, and 150 $\mu g L^{-1}$) were much greater than what is considered environmentally relevant to clearly quantify the relationship between these endpoints. To quantify feeding behaviour of the bass, the time to eat prey (live fathead minnows) was recorded on 3-day intervals of exposure. The study revealed a

strong negative relationship between serotonin levels in the brain and time to capture the fathead minnows. The levels of both 5-HT and its metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA), were significantly decreased with FLX exposure. An unusual effect of FLX in fish also found in this study was abnormal positioning in the water column; that is, the bass tended to be more associated with the surface of water as compared to fish that were unexposed (Gaworecki and Klaine, 2008). This behavioural effect of FLX could have an impact on a fish's ability to avoid predation.

Similarly, Mennigen et al. (2010b) examined the effects of FLX exposure on food intake, energy metabolism, and weight gain by exposing adult goldfish to two aqueous doses (540 ngL^{-1} and 54 μgL^{-1}) for 28 days. The study found that at the higher concentration, both food intake and weight gain were inhibited compared to the negative control group ($p<0.001$; Mennigen et al., 2010b). At the lower concentration, they found a significant change in energy metabolism through the observation of a decrease in plasma glucose concentration ($p<0.01$; Mennigen et al., 2010b). The authors concluded that there was a correlation between increased clearance of glucose by the muscle and a decrease in gluconeogenesis in the liver that demonstrated the alteration in energy metabolism when the fish were exposed to the low treatment only. The effect of FLX on energy metabolism could be related to serotonin's involvement in carbohydrate metabolism as well as its role in regulation of glucose homeostasis in neurotransmission networks of the hypothalamus.

A study conducted by Dorelle et al. (2020) looked at the effects of IP-injected FLX on the food intake of *Cichlasama dimerus*, a cichlid fish from Argentina, as well as other endpoints including liver histology and energy metabolism. Every 24 hours for five days, groups of adult cichlids (n=6) were injected with a saline vehicle control, 2 μgg^{-1} or 20 μgg^{-1} FLX. These doses were selected based the concept of the read-across approach proposed by Rand-Weaver et al.

(2013) and were aiming to fall within the human therapeutic range (91-302 $ngmL^{-1}$) (Margiotta-Casaluci et al., 2014). The fish exposed to the highest FLX dose showed a significant reduction in consumption of food pellets as they failed to consume any pellets throughout the duration of the study ($p=0.0035$) (Dorelle et al., 2020). However, due to large variability among individuals, there was no statistically significant change in body mass in the high dose group. No change in food intake nor body mass was observed in the low FLX dose group. Liver histology analysis revealed cytoplasmic acidophilia in hepatocytes, a dose-dependent reduction in hepatocyte area. Within the liver tissue, there was a dose-dependent trend of decreasing glycogen and lipid content, though differences were not statistically significant. This decrease indicated mobilization of stored glycogen reserves for the short-term response when fasting occurs. When fasting persists, mobilization of hepatic lipid and protein reserves is expected. The authors claim that the results obtained from this study suggested that FLX can produce an anorectic effect in *C. dimerus*; however, further testing is needed to assess environmentally relevant exposure concentrations and longer duration (Dorelle et al., 2020).

Within the current available literature, it is difficult to draw conclusions on the effects of FLX at the population level to fishes (McDonald, 2017). To relate a mechanism of action with population-level effects, research must generate dose-response relationships for effects that may result in significant changes in behaviour or physiology that led to an organism being unable to fulfill its ecological role. Endpoints to have such implications could be, for example, reduced ability to capture prey or reproductive success; however, these effects would be difficult to extrapolate from a lab setting to field scenarios.

1.6.3 Molecular & Biochemical Effects

An analysis of the effects of xenobiotics on the expression of genes involved with regulating key physiological processes can help elucidate their mechanism of toxicity. However, research on molecular and biochemical pathways of FLX in fishes is relatively limited in the current literature. Only in recent years have researchers begun to generate information on effects at the genetic and molecular level with the aim to inform adverse effects observed in aquatic organisms. For example, a few recent studies have shown that FLX can cause alterations in the expression of genes involved with stress- and behaviour-linked pathways, lipid and amino acid metabolic processes, and steroid biosynthesis (Mercier et al., 2004; Wong et al., 2013).

An investigation by Park et al. (2012) involved an analysis of global gene expression in larval zebrafish upon the exposure to two concentrations of FLX (25 and 250 μgL^{-1}) for 96 hours. Their results revealed a total of 288 differentially expressed genes due to the exposure of 25 μgL^{-1} FLX, 67% of which were down-regulated. When exposed to 250 μgL^{-1} FLX, a total of 131 genes were differentially expressed with 53% down-regulation. This suggests that lower concentrations of FLX can have important effects on gene regulation in larval zebrafish. The authors suggested that this is an example of a hormesis-like response which may be driven by overcompensation at the low-dose exposure; however, more experimentation would be necessary to establish a dose-response curve of FLX. Two down-regulated genes were identified that were recognized to be involved in the regulation of stress response: myogenin and FKBP5, the binding protein of immunosuppressant, FK506. Other genes affected by FLX exposure were involved in transcription regulation, metabolism, protein folding, signal transduction, transport, and DNA repair. Interestingly, their gene ontology analysis found that among the biological pathways most affected was cholinesterase activity indicating that SSRIs also have the potential to inhibit

acetylcholinesterase (AChE) activity, and therefore, they may contribute additively to other AChE-inhibiting pollutants such as certain pesticides that may be found in the same waterways. Previous research has shown that SSRIs can inhibit AChE activity in humans (Müller et al., 2002); however, further investigation is required to determine whether the enzyme is also influenced in fish.

Cunha et al. (2018) aimed to address the lack of knowledge regarding the interaction of FLX with the neurotransmitter system. To achieve this, they analyzed the mRNA transcription of serotonin, dopamine, and adrenergic transporters and receptors in early stages of development of zebrafish embryos via qPCR. The study began with zebrafish embryos (1 hpf) exposed to a range of FLX concentrations (0.0015 – 0.8 µM) for 80 hours. They reported a clear downregulation in the transcription of serotonin transporters and receptors at all test concentrations. This change has been attributed to the increase of 5-HT accumulation in the synaptic clefts by repeated inhibition of the 5-HT receptor (Gomez et al., 2015; Lesemann et al., 2012). This decrease in mRNA could affect neurotransmission and provoke behavioural changes as described in previous studies (Painter et al., 2009; Weinberger II and Klaper, 2014). Additionally, the serotonergic system plays an important role in vertebrate embryo development (Buznikov et al., 2001). Because this study was conducted at environmentally relevant concentrations of FLX, the authors suggested that embryonic development is an important endpoint to FLX exposure and should be further studied. In the adrenergic system, only at the higher concentrations were there any observations of a down regulation pattern (Cunha et al., 2018). As for the dopaminergic system, the transcription results also displayed a down-regulation pattern throughout the dosing range. A decrease in mRNA of the dopaminergic receptors and transporters could have a negative impact on functions known to be controlled by this system – for example, cognition, locomotor activity, motivation and reward, attention, and learning (Beaulieu and Gainetdinov, 2011).

In addition to analyzing behavioural effects between the pair of dominant and subordinate male zebrafish as described above (see section 1.4.1), Theodoridi et al. (2017) also conducted an analysis of expression of genes involved in serotonergic system regulation by performing qPCR assays on the brain tissue of zebrafish. The authors made comparisons between the control and treated groups as well as between the dominant and subordinate zebrafish. Treatment of FLX resulted in the upregulation of the 5-HT receptor 2B in both the dominant and subordinate males. Furthermore, mRNA expression levels encoding for the glucocorticoid receptor, mineralocorticoid receptor, *c-fos,* hypocretin/orexin, and brain-derived neurotrophic factor were significantly upregulated in the brains of FLX-treated dominant males. In the subordinates, only the expression of neurotransmitter transporter 4a showed an upregulation compared to the control. Interestingly, the mRNA expression levels of *c-fos* in the dominant and subordinate males had a reversed response due to FLX treatment. In the dominants, a 25-fold increase in expression and in subordinates, a 5-fold decrease. Because *c-fos* is an immediate-early gene, it has been used previously as an indirect marker of neuronal activity. The authors concluded that this brain activity is associated with the offensive and defensive aggression observed in the dominant and subordinate fish.

Because the molecular target of FLX is conserved across vertebrate species, some researchers have utilized aquatic organisms as models for assessing the potential human health consequences via assessment of cDNA microarray-based expression profiling. For example, a study by Thomas et al. (2012) utilized fathead minnows as a model for testing enrichment of gene sets known to be involved in human neuronal development, regulation, and growth. Their experimental design consisted of five juvenile fathead minnows designated to a control and 10 μgL^{-1} FLX in triplicate for 18 days. The FLX treatment resulted in the downregulation of 31 out

of a set of 75 genes involved in neurotransmitter binding, which is likely due to a decrease in brain concentration of the 5-HT receptor. The authors concluded that these expression patterns indicate enrichment of biological processes involving neuronal differentiation and organization.

Recent research aimed to demonstrate that a multi-omics toxicological approach using sensitive animal models can help characterize the toxicological relevance of acute low-dose chemical exposures. Huang et al. (2017) assessed perturbations in both the metabolome and transcriptome of zebrafish larvae exposed from 96 to 120 hours post fertilization to environmentally relevant concentrations of FLX (0.004, 0.4, & 4 μM). To identify molecular interactions and biological relevance, multi-omic responses were evaluated independently and then integrated. Compared to the control group, 36 metabolites and only 2 gene transcripts were significantly affected (Huang et al., 2017, 2016). The more prominent metabolomic effects compared to transcriptomic effects may suggest a more significant contribution from post-translational impacts. The metabolome profile indicated important changes in cellular processes such as: sphingolipid metabolism, β-oxidation of fatty acids, and biosynthesis of proteins and phospholipids (Huang et al., 2017). Additionally, as suggested by a reduction in cellular amino acid concentrations, FLX treatment also targeted aminoacyl-tRNA biosynthesis, which could have implications in numerous downstream pathways. The study revealed an increased gene transcript expression of emopamil binding protein, an endoplasmic reticulum membrane protein involved in cholesterol production. This evidence supports an effect of FLX on cholesterol biosynthesis, which may have implications in serotonergic neurotransmission.

Fluoxetine is administered in a racemic mixture where both the R and S isomers can effectively inhibit the 5-HT transporter and cause an anxiolytic response. In addition to modulation of serotonin levels, SSRIs have shown to also affect production of allopregnanolone,

a modulator of the gamma-aminobutyric acid (GABA) receptor, and various other neuropeptide levels such as neuropeptide Y, oxytocin, and arginine vasotocin/vasopressin (AVT/AVP) (Wong et al., 2013). An experiment testing FLX exposure on male bluehead wrasse found a significantly lower expression of AVT mRNA expression brain tissue relative to the control group (Semsar et al., 2004). In species exhibiting territoriality among males, the AVT/AVP system has a large influence on dominance relationships and aggressive and reproductive behaviours (Semsar et al., 2004).

Because SSRIs appear to affect many neurotransmitter systems in addition to the serotonergic system, understanding the full extent of changes in molecular mechanisms of FLX exposure has become a recent topic of research. Wong et al. (2013) used RNA-sequencing to test for whole brain differential expression of protein-coding genes of racemic FLX-treated male zebrafish. The fish were exposed chronically to 100 μgL^{-1} racemic FLX, stereoisomers R or S, or a solvent control. Using quantitative reverse transcriptase PCR, they assessed the expression of nine genes known to be associated known to either be associated with anxiety-like behaviour or shown more than two-fold differential expression. Two neuropeptides, isotocin and neuropeptide Y, showed significantly higher RNA expression in FLX-treated zebrafish relative to the control. Isotocin is the teleost homolog to the mammalian oxytocin and may play an important role in reproduction whereas neuropeptide Y is associated with reduced anxiety (Lister et al., 2009; Wong et al., 2013). The study also revealed down-regulation of the RNA coding for urocortin 3, an anxiogenic hormone, and prolactin, a hormone known to be associated with stress coping in fish. The gene coding for the GABA transporter was significantly down-regulated in FLX-treated fish, which is also consistent with a reduction in stress and anxiety. Global transcriptome analysis revealed 167 and 244 genes were up- and down-regulated, respectively. Using Kyoto

Encyclopedia of Genes and Genomes (KEGG) to analyze changes in the global transcriptome, the authors found a large representation from amino acid, lipid, and steroid hormone pathways.

Information regarding the effect of FLX on expression of genes related to oxidative stress, stress and anxiety, and major neurotransmission in fish is still relatively limited in the scientific literature. Aiming to address these limitations, Parolini et al. (2019) investigated changes of expression in genes encoding for neurotransmitter transporters and their relation to alterations in the swimming behaviour of zebrafish larvae. Two aqueous concentrations of FLX were examined, 50 ngL^{-1} and 500 ngL^{-1}, over a 96-hour exposure period, post hatch. When examining the behavioural response in a touch-evoked response assay, they found that when exposed to either concentration of FLX there was a significant decrease in swimming behaviour, measured as distance travelled, as compared to the negative control ($p<0.001$) (Parolini et al., 2019). In addition, expression of anxiety-related behaviour genes was also examined via qRT-PCR. A statistically significant increased expression of isotocin (*oxtl*) at both tested FLX concentrations ($p=0.004$) (Parolini et al., 2019). Isotocin is known to have a role in a variety of behaviours related to stress and anxiety. Both tested concentrations resulted in an upregulation of expression of genes encoding for both orthologues of the serotonin transporter (*slc6a4a* and *slc6a4b*; $p<0.001$) and GABA transporters (*slc6a11*; $p=0.001$). The authors hypothesize that the increase in extracellular 5-HT may be related to the increase in isotocin; and, in turn, due to this influx of 5-HT, there was a homeostatic upregulation of the serotonin transporter genes. When analyzing the differential expression of genes encoding for key antioxidant enzymes, Parolini et al. (2019) found a significant upregulation of two orthologues encoding for superoxide dismutase (*sod1*; $p<0.001$ & *sod2*; $p=0.021$) and catalase (*cat*; $p=0.002$). The increase in *sod1* and *sod2* expression suggests

that exposure to FLX induced the production of superoxide anion. Likewise, the induction of *cat* suggests the cellular defence against hydrogen peroxide toxicity.

1.7 Project Rationale

Currently, there is limited data on the molecular toxicity pathways triggered by the exposure to FLX in fathead minnows. Very few studies have been performed using omics techniques to elucidate molecular responses of FLX exposure, and, to my knowledge, there is no literature regarding a multi-omics approach in utilizing transcriptomics and proteomics. Additionally, due to the complexity of the physiology of the serotonergic system and the shortcomings of the read-across hypothesis, molecular toxicity pathways are not well understood in teleosts. Very few examples exist in current scientific literature on an established toxicity pathway of FLX, especially utilizing fathead minnows as a model species. In terms of apical endpoints, studies conducted that examine the effects of FLX on fecundity have not reached a consensus. For these reasons, and in the context of increasing concern of SSRI contamination in the aquatic environment, it is important to address the lack of information currently available on predictive toxicity pathways of FLX.

The overall goal of this thesis research was to gain a better understanding of the molecular toxicity pathways of FLX exposure on fathead minnows and provide much needed data on responses across levels of biological organization, from the transcriptome and proteome to whole animal. Research was first focused on evaluating adverse outcomes of long-term exposure to FLX including assessing changes in morphometric parameters, fecundity, and liver histopathology. I also conducted a second experiment to examine molecular pathway dysregulation following a short-term exposure to FLX. Studies were conducted with the laboratory teleost model *P. promelas* because of (1) the standardized exposure protocol in the adult life stage and ease of

handling, (2) the abundance of scientific literature previously established on apical endpoints, and (3) the accessibility of the sequenced and annotated genome of the species.

The aim of this research was to investigate and validate key molecular toxicity pathways that are predictive of apical outcomes induced by exposure to FLX in adult fathead minnows.

To accomplish this goal, this study examined the key molecular toxicity pathways induced after 96 hours of exposure to FLX in *P. promelas* by assessing the dysregulation of the global transcriptome and proteome. Following, this research assessed apical level effects in *P. promelas* that are well described and of regulatory relevance following a 21-day exposure to environmentally relevant concentrations of FLX. Finally, through characterizing the relationship between the response at each level of biological organization, assembly of a comprehensive toxicity pathway to determine if key molecular signatures can be predictive of adverse outcomes of regulatory relevance.

1.8 Research Objectives and Hypotheses

The overall objective of this thesis research was to identify and validate key molecular toxicity pathways that are predictive of apical outcomes induced by exposure to FLX in adult *P. promelas* as a model species for teleosts. This was accomplished by identifying biological pathways that are altered with short-term exposure to FLX and relating these molecular changes to higher-level responses in the following chronic exposure. This information was then used to establish predictive toxicity pathways of FLX and identify gene expression patterns that can predict adverse outcomes of regulatory relevance.

Specific objectives:

1) Determine if short-term (21-day) exposure to FLX alters apical responses in adult *P. promelas* including morphology, fecundity, or liver histopathology

H0: There are no statistically significant effects on gross morphology, fecundity, or liver histopathology in adult P. promelas *when exposed to graded concentrations of FLX for 21 days*

2) Determine if short-term (96hr) exposure to FLX alters molecular response patterns in adult *P. promelas* using whole transcriptome and proteome analyses

   H0: There are no statistically significant differences in molecular response patterns in the transcriptome or proteome, of adult P. promelas *exposed to graded concentrations of FLX compared to the control group*

3) Characterize relationships between molecular response patterns and physiological, histopathological, and apical effects to determine if gene expression patterns can be identified that are predictive of adverse outcomes of FLX exposure in adult *P. promelas*

   H0: No concordance can be identified between molecular responses and apical response patterns in P. promelas *following exposure to graded concentrations of FLX*

CHAPTER 2: CHARACTERIZING TOXICITY PATHWAYS OF FLUOXETINE TO PREDICT ADVERSE OUTCOMES IN ADULT FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

Preface

This study assessed the toxicity of an emerging aquatic contaminant of concern, fluoxetine, in adult fathead minnows (*Pimephales promelas*), a model fish species. A short-term reproductive assay was conducted to characterize response in morphometrics, fecundity, and histopathology of fathead minnows exposed to the same concentrations of FLX. Proteomic and transcriptomic responses were also evaluated after 96 hours of FLX exposure with the aim of identifying key molecular signatures that may be predictive of apical level outcomes. This chapter was prepared as a manuscript and was submitted for publication to the peer-reviewed scientific journal *Science of the Total Environment*.

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Author contributions:

Carly Colville (University of Saskatchewan) designed the study, collected, processed, and analyzed samples, performed statistical analyses, prepared figures and tables, and drafted manuscript.

Alper James Alcaraz (University of Saskatchewan) helped with study design, sample collection, advised on statistical analyses, provided feedback on manuscript.

Derek Green (University of Saskatchewan) helped with study design, sample collection, advised on statistical analyses, provided feedback on manuscript.

Bradley Park (University of Saskatchewan) processed and analyzed histopathology samples and provided feedback on manuscript.

Jeff Xia (McGill University) developed software for data workflow and provided feedback on manuscript.

Othman Soufan (St. Francis Xavier University) developed software for data workflow and provided feedback on manuscript.

P. Hruška (Masaryk University) processed and analyzed samples for proteomics and provided feedback on manuscript.

D. Potěšil (Masaryk University) software development and supervised sample processing and analysis for proteomics and provided feedback on manuscript.

Z. Zdráhal (Masaryk University) supervised sample processing and analysis for proteomics and provided feedback on manuscript.

Doug Crump (Environment and Climate Change Canada) provided scientific input as a project principal investigator, project administration, provided feedback on manuscript, and provided funding required to conduct the research.

Nil Basu (McGill University) provided scientific input as a project principal investigator, project administration, provided feedback on manuscript, and provided funding required to conduct the research.

Natacha Hogan (University of Saskatchewan) provided scientific input as a project principal investigator, project administration, provided feedback on manuscript, and provided funding required to conduct the research.

Markus Hecker (University of Saskatchewan) designed study and methodology, provided scientific input and guidance, provided feedback on manuscript, and provided funding required to conduct the research.

Abstract

Current ecotoxicity testing programs are impeded as they predominantly rely on slow and expensive animal tests measuring adverse outcomes. Therefore, new approach methodologies (NAMs) increasingly involve short-term mechanistic assays that employ molecular endpoints to predict adverse outcomes of regulatory relevance. This study aimed to elucidate the application of NAMs in adult fathead minnows using fluoxetine (FLX) as a model compound. Fish were exposed to three FLX concentrations (measured: 2.42, 10.7, and 56.7 μgL^{-1}) and a control. After 96 hours, molecular toxicity signatures were characterized using proteomics and transcriptomics analyses in livers and brains of a sub-set of fish. The remaining fish were sampled at 21 days and assessed for liver histopathology and morphometric measurements. Fecundity was monitored throughout the study. In the livers, 56.7 μgL^{-1} FLX caused enrichment of PPAR signalling in the proteome and fatty acid-related pathways in the transcriptome, potential upstream responses that led to lipid-type vacuolation of hepatocytes, observed via histopathology. Upregulated genes in the brain suggested alterations in serotonin-related signalling processes and reproductive behaviour, which may explain the observed significant decrease in fecundity. While the relationships between molecular responses and adverse outcomes remain complex, this research provided important insights into the mechanistic toxicity of FLX.

2.1 Introduction

Critical challenges facing environmental risk assessment include the large number of chemicals that currently require toxicity testing (e.g., upwards of 350,000; Wang et al., 2020) and the continuous increase of new contaminants entering the environment each year (e.g., ~500-1000; Basu et al., 2019). Current ecotoxicity testing programs mandated by regulatory agencies predominantly rely on *in vivo* tests measuring traditional adverse outcomes such as mortality, growth, disease, reproductive failure, or developmental dysfunction (Villeneuve and Garcia-Reyero, 2011). However, there are significant concerns regarding these methods because of their high cost and requirement of long-term and time-consuming exposure experiments with live animals. The development of new approach methodologies (NAMs) aims to examine mechanistic toxicity at the cellular, tissue, or whole organism level while improving the efficiency, economy, and reliability of data collection and reducing the need for lengthy live animal testing (Basu et al., 2019; Villeneuve and Garcia-Reyero, 2011). These approaches include *in silico*, *in vitro*, embryonic models and short-term mechanistic studies involving transcriptomics, proteomics, metabolomics, and other high-throughput or big data technologies. Most NAMs postulate that understanding the specific mechanism by which a contaminant causes an adverse response enables effective prediction of the most likely physiological adverse outcome. In particular, the inclusion of multi-omics analyses has the potential for improved elucidation of the underlying molecular mechanism(s) that lead to a biological/toxicological response, and therefore enhances prediction of adverse outcomes in whole animals in the absence of morphological effects (Huang et al., 2017). To this end, attempts to meet contemporary testing requirements could benefit substantially from well-established, short-term, mechanistic assays for ecological species of interest.

Aquatic ecosystems are exceptionally vulnerable to chemical released through wastewater effluent or industrial activities (e.g., pharmaceuticals and personal care products). Fluoxetine (FLX), the active ingredient in Prozac® (Eli Lilly), is one of the most frequently prescribed anti-depressants and is increasingly prevalent in wastewater treatment effluent-receiving aquatic ecosystems (Kreke and Dietrich, 2008; Pratt et al., 2017). The median concentration of FLX in effluent-receiving surface waters is 17.8 ngL^{-1}; however, concentrations upwards of 596 ngL^{-1} have been reported (Hughes et al., 2013). FLX is a selective serotonin reuptake inhibitor (SSRI) which targets the evolutionarily and functionally conserved serotonin transporter; therefore, FLX can affect nontarget species, including fish, with similar efficacy (Amador and McDonald, 2018; McDonald, 2017). Several studies have reported adverse effects of FLX exposure in fish: altered anxiety and social and mating behaviour (Margiotta-Casaluci et al., 2014; Theodoridi et al., 2017; Weinberger II and Klaper, 2014), altered reproductive fitness (Henry and Black, 2008; Mennigen et al., 2010a; Prasad et al., 2015; Schultz et al., 2011), decreased predator avoidance (Painter et al., 2009), and decreased ability to catch prey (Gaworecki and Klaine, 2008).

Fish are key vertebrate models for toxicity testing because they are of specific ecological concern. That is, they are often present in contaminant-receiving surface waters and several fish species have demonstrated high sensitivity to xenobiotic exposure. In particular, the fathead minnow (*Pimephales promelas*) is a model fish species and has been widely used in ecotoxicity testing for decades. Fathead minnows are well-characterized regarding toxicological responses to an abundance of chemical contaminants across a range of biological endpoints, including biochemical, physiological, reproductive, and behavioural outcomes. In addition, recent efforts have been made to expand this breadth of knowledge to the molecular level through sequencing

and annotating of the genome of the fathead minnow, a key initiative to support toxicogenomic investigations (Martinson et al., n.d.).

Despite the widespread use of FLX and its well-defined adverse effects in fish species, there is limited data available that conclusively link molecular toxicity events (Cunha et al., 2018; Huang et al., 2017; Park et al., 2012; Thomas et al., 2012; Wong et al., 2013) with physiological effects across multiple levels of biological organization. Given the dynamics of gene expression, there is substantial uncertainty with interpreting this type of data regarding the potential for downstream adverse outcomes. Integration with higher-level organizational analyses such as proteomics could address these uncertainties, and potentially help in building toxicity pathways to support environmental risk assessment. Therefore, the objectives of this study were to 1) determine if acute exposure to FLX alters molecular response patterns in *P. promelas* using whole proteome and transcriptome analyses, 2) characterize apical level effects (morphometry, fecundity, and histopathology) of regulatory relevance using a 21-day short-term reproductive assay (OECD, 2012), and 3) establish molecular toxicity pathways for FLX in *P. promelas* that can inform biological outcomes of regulatory relevance through integration of omics, histological and apical outcome data.

2.2 Materials & Methods

2.2.1 Test Organisms

Adult fathead minnows (>6 months of age) were obtained from a standing culture maintained in the Aquatic Toxicology Research Facility (ATRF) at the Toxicology Centre at the University of Saskatchewan (SK, Canada). All fish used in this study were handled in accordance with the University of Saskatchewan's Animal Research Ethics Board (protocol #20160090) and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.2.2 Test Chemical Preparation

Fluoxetine hydrochloride powder (CAS# 56296-78-7, purity: ≥98%) was obtained from Sigma-Aldrich (Oakville, ON). FLX stock solution (5 gL^{-1}) was prepared by dissolution in Nanopure™ water and stored at 4 °C, from which working stock solutions (39.0, 156, and 625 mgL^{-1}) were prepared. Working stock solutions were added to the reservoirs which fed the flow-through aquaria to achieve final nominal exposure concentrations of 7 μgL^{-1} ("low"), 28 μgL^{-1} ("medium"), and 112 μgL^{-1} ("high").

2.2.3 FLX Exposure Experiment

Exposures took place in the ATRF and followed the *OECD 229: Short Term Reproductive Assay* procedure (OECD, 2012). In brief, two parallel studies were conducted; a short-term exposure experiment (96 hours; n=5 replicates per treatment group) to generate tissues samples of omics analyses, and a sub-chronic exposure experiment (21 days; n=5; Appendix A) for histopathological and apical outcome assessments. The three FLX treatment levels were assessed along side a water control group under the same experimental conditions. The experiments were conducted under flow-through conditions with 3x exposure solution replacement (v/v) per day in 20L aquaria maintained at 25°C±2°C. Two male and three female fish were allocated to each aquarium. The exposure period was preceded by 18 days of acclimation and a 10-day baseline period until all aquaria had consistently breeding minnows and to ensure proper sex ratios. Following baseline, aquaria were ranked according to the calculated fecundity and randomly assigned exposure condition with equal distribution of rank among exposure conditions. Water quality parameters were monitored daily in eight randomly selected aquaria, including temperature, dissolved oxygen, conductivity, and pH, using a YSI Quatro Professional Plus multiparameter field cable (Yellow Springs, USA); and ammonia, nitrates, nitrites, hardness, and

alkalinity, using testing kits (Mars Fishcare, Chalfont, USA; LaMotte, Chestertown, USA; Table E.1).

Throughout the exposure, embryos were collected from breeding tiles, counted, and photographed, twice daily. Observations of spawning were recorded for each experimental unit, and cumulative fecundity was calculated as the number of embryos spawned per surviving female per day. After 96 hours of exposure, fish from the short-term exposure experiment were euthanized for subsequent tissue collection. Fish were fasted 24 hours prior to sampling. Observations of abnormalities were recorded for each fish and morphometric measurements were taken including wet body mass (to the nearest 0.001 g) and standard length (to the nearest 0.001 cm). Liver and brain tissues were excised from each fish, weighed, and flash-frozen in liquid nitrogen before being stored at -80°C for subsequent transcriptomic and proteomic analyses. On day 21 of exposure, fish from the remaining experiment were euthanized after fasting for 24 hours. Fish were subjected to the same morphometric measurements as described for the short-term experiment. Whole viscera were collected from one male fish per experimental unit for histopathological analysis of the livers. To evaluate changes in somatic physiological status, condition factor (K; Eq. 1) and hepatosomatic index (HSI; Eq. 2) were calculated (Froese, 2006).

$$K = W_{total} \times 100 / {L_{standard}}^3 \qquad (1)$$

$$HSI = W_{liver} / W_{total} \qquad (2)$$

Where: Mass (g) is represented by W and standard length (cm) is represented by L.

2.2.4 Analysis of FLX Exposure Concentrations

Water samples were collected from each experimental unit at four time points (exposure days 1, 6, 12, and 21) and pooled at each time point for FLX analysis. Samples were preserved using 2 drops of chloroform per liter of sample, and initially kept at -20°C for 24 hours and

subsequently transferred to -80°C for storage until analysis. Samples were sent to SGS AXYS Analytical Services Ltd. (Sidney, BC, Canada) for analysis of FLX, following the methods described in Long et al. (2013) (Appendix B). Briefly, water samples were extracted by solvent-phase extraction (SPE) and spiked with deuterated fluoxetine (d5-FLX; CDN Isotopes, QB, Canada). FLX was then quantified using a Waters 2690 HPLC (Waters, MA, USA), equipped with a Xterra MS C18 column (Waters; 10.0 cm, 2.1 mm i.d., 3.5 µm particle size), and connected to a Micromass Quattro Ultima MS/MS (Waters). Concentrations were quantitated based on a seven-point weighted linear calibration and response relative to deuterated (d5) FLX.

2.2.5 Liver Histopathology

Histopathological analysis was performed on a randomly selected male fish from each aquarium of the water control and the high treatment groups (n=5 per exposure condition). Viscera samples were submerged and fixed in CalEx-II (Fisher Scientific) for 48h then transferred to 70% ethanol for storage until processing. The fixed samples were dehydrated in alcohol, cleared in xylene, and infiltrated with molten paraffin using a Belair RVG/1 Vacuum Tissue Processor. The samples were then embedded in paraffin blocks and sectioned longitudinally (5 µm thick). Representative sections were taken at six levels through the viscera at 150 µm intervals. The sections were affixed to glass slides and stained with Harris' hematoxylin and eosin. Histopathological sections were analyzed using a Zeiss Axiostar Plus microscope, interfaced with a Lumenera INFINITY1-1M digital camera, and Lumenera Infinity Analyze software (release 6.5.4). Histopathological changes in livers were assessed visually for changes in staining properties, vacuolation, cell size and structure, and nuclear appearance.

2.2.6 Proteomics

Proteomic analyses were conducted on liver samples excised from male fathead minnows (two individuals pooled per replicate; n=5 replicates per experimental condition) from facility water control and high conditions, following the methods described in Alcaraz et al. (2021), with minor modifications. The high FLX treatment group was selected for analysis with the aim of capturing molecular responses at the proteomic level as this concentration has been shown to elicit the most significant responses in a parallel study conducted with early life stage fathead minnows and rainbow trout by our group (Alcaraz, personal communication). Briefly, samples were pooled and then processed using filter-aided sample preparation (FASP), and digested peptides were analyzed using a non-targeted, data-dependent LC-MS/MS using an Ultimate 3000 RSLCnano system (SRD-3400, NCS-3500RS CAP, WPS-3000 TPL RS; Thermo Fisher Scientific) interfaced with a Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

Raw mass spectrometric data files were analyzed using MaxQuant (Cox and Mann, 2008; Tyanova et al., 2016) using the *P. promelas* reference proteome (NCBI Acc# GCA_016745375.1; 48,456 sequences Martinson et al., n.d.) and the cRAP contaminant database (November 2018; 112 sequences). Peptide and protein identifications with a false discovery rate (FDR) <1% and at least one razor ("unique") peptide were selected for downstream analyses. The data were filtered to at least one measured intensity value in at least one replicate of either experimental group. Loess normalization strategy was applied to all log2 transformed protein group intensities across samples. The missing intensity values were subsequently imputed using imp4p (Gianetto et al., 2020) accordingly to experimental groups. Relative protein group abundance was evaluated using LIMMA (Ritchie et al., 2015), and differences were considered significant at a cut-off FDR

(Benjamini-Hochberg, BH) of ≤0.05. Differential abundance analyses were done in KNIME (Berthold et al., 2008). Raw data were deposited in the ProteomeXchange Consortium via the Proteomics Identifications (PRIDE) partner repository (Acc#PXD028287).

2.2.7 Transcriptomics

Transcriptomic analyses were conducted on liver and brain tissues excised from male fathead minnows from the facility water control, medium, and high FLX experimental conditions (n=5; Appendix C). Due to logistical and funding restraints, only the medium and high FLX treatment group were selected for analysis with the aim of capturing molecular responses at the transcriptomic level. Total RNA was extracted from tissue samples of individual fish using the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) in a QIACube instrument (Qiagen). Concentrations of RNA were evaluated using a QIAxpert instrument (Qiagen), and equal masses of RNA were pooled from individuals (i.e., two male fish) within each experimental unit (n=5). RNA-mass-equivalent pooled samples were assessed for RNA Integrity Number (RIN) in an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Samples with RIN ≥9 were sent to the Genome Quebec Innovation Centre (McGill University, Montreal, QC, Canada) for mRNA sequencing using an Illumina NovaSeq instrument (Illumina, San Diego, CA, USA). Raw RNA-Seq FASTQ files were assessed for quality, trimmed (Krueger, n.d.) to a minimum Phred score of 20 and a minimum sequence length of 35 bases per paired read using Trim Galore!, and aligned to the *P. promelas* reference transcriptome (NCBI Acc# GCA_016745375.1) (Martinson et al., n.d.) using Kallsito quant (Bray et al., 2016) in the EcoToxXplorer Galaxy server (https://galaxy.ecotoxxplorer.ca/). Raw count data were filtered to remove gene features with less than 20 counts per million in at least five samples. Differential expression analysis was performed using the DESeq2 R package (Love et al., 2014). Genes were considered significantly

differentially expressed at FDR-adjusted p-value ≤0.05 relative to the water control group. Raw FASTQ files are available through NCBI GEO (Acc# GSE182913).

2.2.8 Enrichment Analysis

Enrichment analyses were conducted using ClueGO (version 2.5.7; Bindea et al., 2009) in Cytoscape (version 3.8.1; Shannon et al., 2003). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases for *Danio rerio* were used to identify and visualize functionally grouped networks of significantly dysregulated features. For proteomics, pathways were built using at least three significantly dysregulated proteins. For transcriptomics, pathways were built using at least two significantly dysregulated genes. Given the large set of features identified in the proteome, the discrepancy of three features instead of two was decided upon to focus the analysis. A right-sided hypergeometrical test with a Bonferroni (step down) p-value correction and a kappa score of 0.4 was used. Terms and pathways identified in the proteome and transcriptome were then compared qualitatively.

2.2.9 Statistics

Morphometric data were tested for a normal distribution using the Shapiro-Wilk test and for homoscedasticity by inspection of residual and QQ plots. Where the assumptions of normality and/or homoscedasticity were violated, data were analyzed using a non-parametric Kruskal-Wallis H test ($p \leq 0.05$). When the assumptions of normality and homoscedasticity were met, data were analyzed using a one-way analysis of variance (ANOVA). For fecundity, the best fit (Hartig, 2020) generalized linear model revealed there was a significant interaction between FLX treatment and time (days), and therefore, the differences in the trendlines of the rate of embryo production per surviving female were examined to determine FLX-induced effects (Appendix D). All

analyses were conducted using GraphPad Prism 9 (San Diego, CA, USA) or RStudio (version 1.4.1717).

2.3 Results & Discussion

2.3.1 Chemical Analysis of FLX

Measured FLX concentrations (nominal: 7, 28, and 112 μgL^{-1}) were 2.42±0.39, 10.7±0.94, and 56.7±3.0 μgL^{-1} in the FLX-treated groups, respectively, while FLX in the controls was 0.017±0.003 μgL^{-1} (Table S.2). This corresponds to 35%, 38% and 51% of nominal concentrations and indicates significant loss of FLX during the exposure. These relatively lower measured concentrations were possibly due to adsorption of FLX to biological matter within the aquaria or bioaccumulation and metabolism by the fish. Similar losses of FLX in aqueous exposures were previously observed in studies by Hazelton et al. (2014) who reported ~50% reduction from nominal levels and Foster et al. (2010) who reported FLX concentration in their low treatment (<0.1 μgL^{-1}) as 0.029 μgL^{-1}, and the concentration for the high treatment (<1.0 μgL^{-1}) as 0.08 μgL^{-1} before water changing and 0.29 μgL^{-1} after water changing. FLX concentrations found in the control group were negligible.

2.3.2 Morphometrics

No significant differences (ANOVA) in morphometric parameters were observed in male or female fish after the 21-day exposure to FLX: condition factor (Male: $F_{3,37}$=0.54, p=0.66; Female: $F_{3,46}$=0.25 p=0.86), hepatosomatic index (HSI; Male: $F_{3,20}$=0.36, p=0.78; Female: $F_{3,25}$=2.50 p=0.082), or wet body weight (Male: $F_{3,37}$=1.19, p=0.33; Female: $F_{3,46}$=0.82 p=0.49; Table S.3). Previous research examining the effect of FLX on these measurements in fish is rather limited, with no literature assessing the same morphometric endpoints in *P. promelas*. Regarding condition factor, current literature demonstrates that FLX exposure does not influence change in

the physical condition of fish (Chen et al., 2018; Foran et al., 2004), which is in accordance with our findings. Varying results are reported in terms of HSI, likely depending on species, FLX concentration, or duration of exposure; for example, studies by Chen et al. (2018) and Mennigen et al. (2010b) observed an increase in HSI whereas no change was observed by Dorelle et al. (2020). Lister et al. (2009) examined wet weight of adult zebrafish exposed to aqueous FLX at comparable levels and found no change in this parameter. The inconsistencies among this study and those previously reported suggest that morphometrics may not be sensitive indicators of adverse response to FLX exposure in fish after short-term exposures such as conducted here. Uncertainty remains in terms of the effect of FLX on the physical fitness of *P. promelas*; however, findings in this study indicate no morphological adversities. Perhaps the 21-day exposure duration was not sufficient to result in observable changes in morphometric parameters of this species. Future investigations should focus on more sensitive and relevant endpoints, such as fecundity.

2.3.3 FLX Exposure Affects Fecundity

The fecundity model yielded an interaction between the FLX treatment and days ($F_{3,1}$=21.83, p=7.08E-05); accordingly, significance was determined by comparing the slopes of the trendlines of the rate of embryos produced. Over the 21-day exposure period, there was a concentration-dependent decrease in fecundity per surviving female per day, which was statistically significant in the low (0.051 [95% CI 0.021 – 0.081], medium (0.075 [95% CI 0.050 – 0.099]), and high treatment groups (0.068 [95% CI 0.043 – 0.093]) compared to the control group (0.119 [95% CI 0.010 – 0.14; $p<0.05$]; Figure 2.1). It is especially important to highlight the reduced reproductive rate in the low (2.42 μgL^{-1}) group. As this was the lowest tested concentration, and considering the observed magnitude of change, the true benchmark dose for this endpoint is likely lower, suggesting potential impacts on the fitness of this species at levels that fall within environmentally

relevant concentrations previously measured in wastewater-receiving aquatic systems (Hughes et al., 2013). An interesting trend across the three FLX-treated groups was the limited deviation from the control slope until the latter half of the exposure period, suggesting a considerable delay for the reduced fecundity response to emerge. SSRIs inhibit the serotonin transporter within minutes of exposure; however, it takes several weeks of treatment to achieve their full effect – a phenomenon well-described in the therapeutic application in humans but not yet in endpoints, such as fecundity, in fish.

Few other studies have analyzed the effects of short-term FLX exposure on the fecundity of fishes, with inconsistent results. A study by Lister et al. (2009) demonstrated that zebrafish exposed to 32 μgL^{-1} FLX for seven days spawned significantly fewer embryos than the control group. In contrast, a study with Japanese medaka exposed to environmentally relevant concentrations of FLX for four weeks yielded no significant change in reproductive output (Foran et al., 2004). Weinberger II and Klaper (2014) determined that four weeks of FLX exposure in fathead minnows impacted male nest preparation, maintenance, and defense behaviour in concentrations as low as 1 μgL^{-1}; however, observed no change in embryos spawned. The differences between these works and the present study could be attributed to exposure concentrations, duration, species, or other experimental factors (Foran et al., 2004).

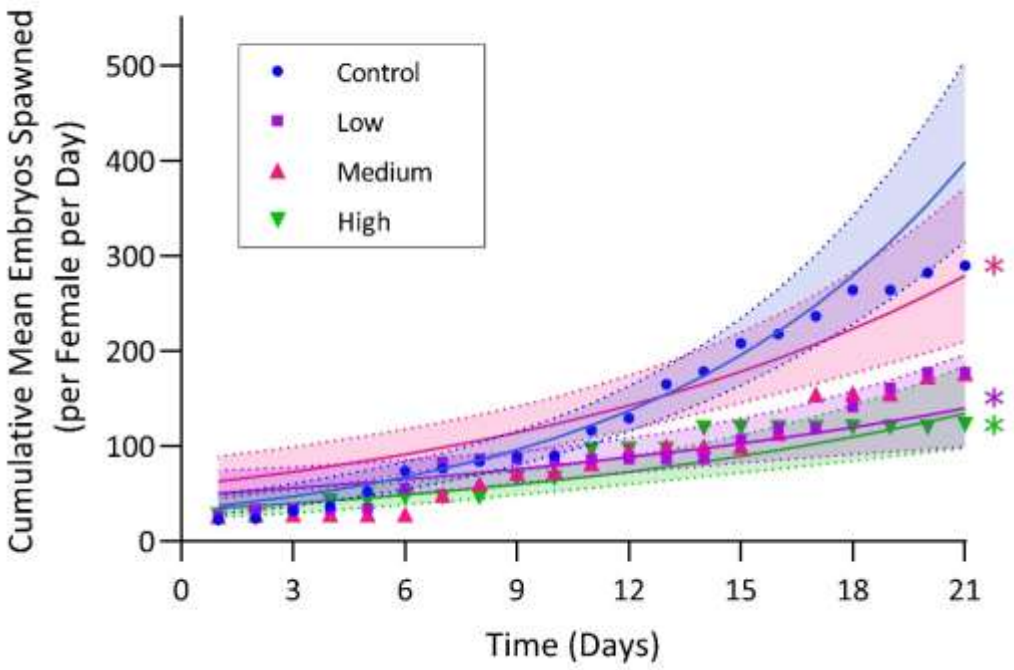

Figure 2.1. Cumulative mean number of embryos spawned by *P. promelas* during 21 days of aqueous exposure to FLX. The four treatment groups are control (blue; 0 μgL^{-1}; n=5), low (violet; 2.42 μgL^{-1}; n=5), medium (magenta; 10.7 μgL^{-1}; n=5), and high (green; 56.7 μgL^{-1}; n=5). The lines represent the best fit slope of the predicted model of the data. Data points represent the cumulative mean embryos spawned. The coloured bands represent the 95% confidence intervals. Asterisks (*) indicate statistically significant difference ($p<0.05$) in the rate of embryos spawned in the treated groups compared to the control group.

2.3.4 Histopathology of the Liver in Response to FLX Exposure

Control fish exhibited livers with normal histological appearance (i.e., cords of hepatocytes interspersed with sinusoids) with relatively uniform spherical nuclei, each containing a single prominent nucleolus. In the FLX-exposed fish, two out of five individuals exhibited a marked increase in lipid-type vacuolation throughout the liver (Figure 2.2); i.e., sharply outlined, clear circular vacuoles that tend to displace the nucleus to the cell periphery (Wolf and Wheeler, 2018). No other histopathological changes were observed. Previous examinations of liver histopathology in FLX-exposed fish showed varied results. In adult male fathead minnows exposed to FLX levels comparable to those of the present study, Schultz et al. (2011) observed no change in the abundance of hepatic adipocytes and vacuolization of hepatocytes. In contrast, FLX-exposed zebrafish displayed histopathological alterations in the liver that may indicate a degenerate process due to metabolic stress; notably, a decrease in vacuolation with exposure to 0.01 μgL^{-1} FLX relative to control (de Farias et al., 2020). Feng et al. (2012) found evidence that FLX induced hepatic lipid accumulation in mice in accordance with molecular pathway perturbations related to altered triglyceride metabolism. Hepatocyte vacuolation can indicate stored energy in the form of lipid, or possibly a sign of degenerative changes in which there is fluid distention of organelles and then an accumulation of free fluid in the cytoplasm (Wolf and Wheeler, 2018). Heavily vacuolated livers, due to the storage of carbohydrates or lipids, can indicate excess energy intake relative to the physical demands of the fish. There is little empirical evidence to suggest that fish with vacuolated livers as observed in our study would function abnormally (Wolf and Wheeler, 2018); however, the present study does indicate altered molecular expression (e.g., PPAR signalling and lipid biosynthetic and metabolic processes; Sections 2.3.5 & 2.3.6) in the liver which may demand further investigation.

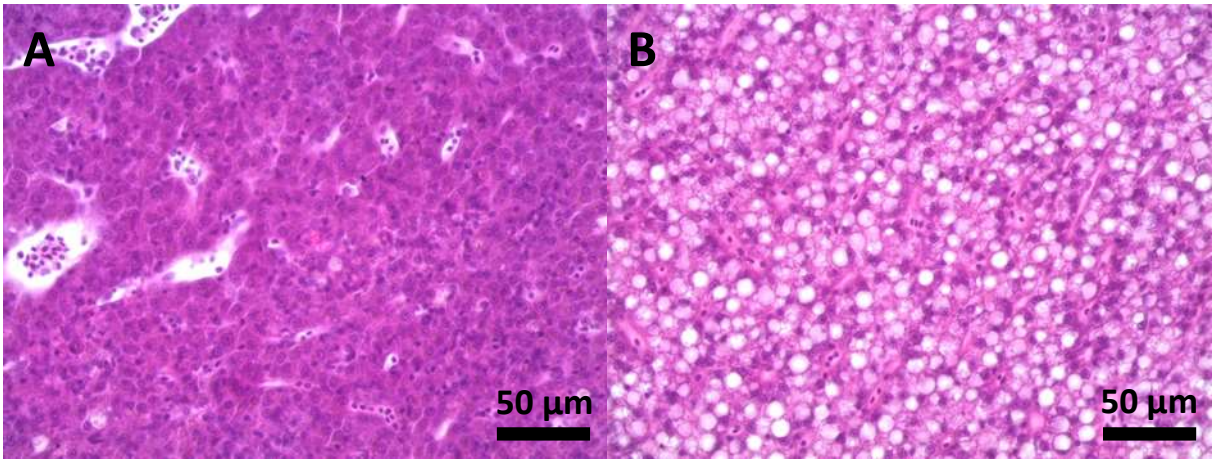

Figure 2.2. Photomicrographs of livers from adult male *P. promelas* (Hematoxylin and Eosin stain). (A) Control, showing little vacuolation. (B) High (56.7 μgL^{-1} FLX) treatment, with abundant lipid-type vacuolation (clear spheres within the hepatocytes), which was observed in 2 of 5 high-treated male individuals. No other histopathological abnormalities were observed. Bar=50 microns.

2.3.5 *Liver Proteomics in Male* P. promelas

Following 96-hour exposure to the high FLX treatment, whole proteome sequencing revealed a total of 419 differentially abundant proteins (DAPs) in the liver tissue of male fish compared to the control group (FDR-adjusted p-value ≤ 0.05). Of the total number of DAPs, 211 were downregulated and 208 were upregulated. Notably, only 314 of the total DAPs were annotated with the current fathead minnow genome. After analysis of pathway dysregulation of these 314 DAPs, 15 GO terms or KEGG pathways were identified in seven distinct groups of biological processes and molecular functions (Figure 2.3; Figure E.2; Table E.4).

Two of the dysregulated KEGG pathways identified in the analysis are involved in cellular signaling: glycerolipid metabolism and PPAR signaling pathway. Products of glycerolipid metabolism are known to function as second messengers in cellular signaling pathways (Ghosh et al., 1997). Peroxisome proliferator-activated receptors (PPARs) are ligand-activated (e.g. polyunsaturated fatty acids and eicosanoids) transcription factors and known regulators of lipid and lipoprotein metabolism, lipid transport, glucose and lipid homeostasis, oxidation, cellular differentiation, and inflammation (Chinetti et al., 2000; Cunha et al., 2013). In addition to its classical neurotransmitter mechanism, serotonin may have a function that is independent of this receptor-mediated function including involvement in lipid metabolism, in accordance with gene expression patterns observed across the transcriptome (Section 2.3.6). Waku et al. (2010) suggests this separate function is mediated by serotonin metabolites that act as endogenous ligands to PPAR. Because the serotonin transporter is inhibited by FLX, this pathway is presumed blocked and may result in the dysregulation of the PPAR signaling pathway observed in the present study. Furthermore, a decrease in PPARα activity would potentially lead to a decrease in lipid clearance

(Cunha et al., 2013) and therefore an accumulation in the liver, as observed in the form of lipid-type vacuolation in the histopathology analysis (Section 2.3.4).

The pathway enrichment in grouped terms including RNA helicase activity, establishment of RNA localization, and translation initiation factor activity is a strong indication that there is dysregulation in transcription and translation processes occurring in the liver of fish exposed to FLX. The enrichment of biosynthetic and metabolic processes of nucleotide sugars, upstream substrates of post-translational modification pathways, further indicates that FLX alters energy-related functions in fish (Hadley et al., 2014; Orellana et al., 2016). These dysregulated processes may also be linked to the differentially enriched term cellular response to xenobiotic stimulus.

Craig et al. (2014) investigated the expression of microRNA (miRNA) in zebrafish larvae exposed to aqueous FLX and found an increased expression profile of miRNA transcripts. miRNAs are important post-transcriptional regulators of mRNA translation and function by binding to the 3'untranslated region (UTR) of mRNA and block translation of the effected gene. In the present study, proteomics analysis revealed pathway enrichment of mRNA 3'-UTR binding, which may be related to the mechanism observed by Craig et al. (2014).

Few studies have been previously conducted using whole proteomic analyses to assess molecular mechanisms of FLX in any model species, with no studies in the current literature using a teleost such as the fathead minnow. Filipović et al. (2017) examined the pathway dysregulation caused by FLX exposure in the cytosolic and non-synaptic hippocampal mitochondria proteomes of adult male rats and found some similar trends to the present study, including the KEGG amino sugar and nucleotide sugar metabolism pathway as well as general metabolic dysregulation, transporter activity, and molecular binding. The present study provided a more in-depth analysis of the effects of FLX on the whole proteome, advancing our understanding of its molecular toxicity

pathways in fish species and providing information for future research in pathway enrichment caused by FLX exposure.

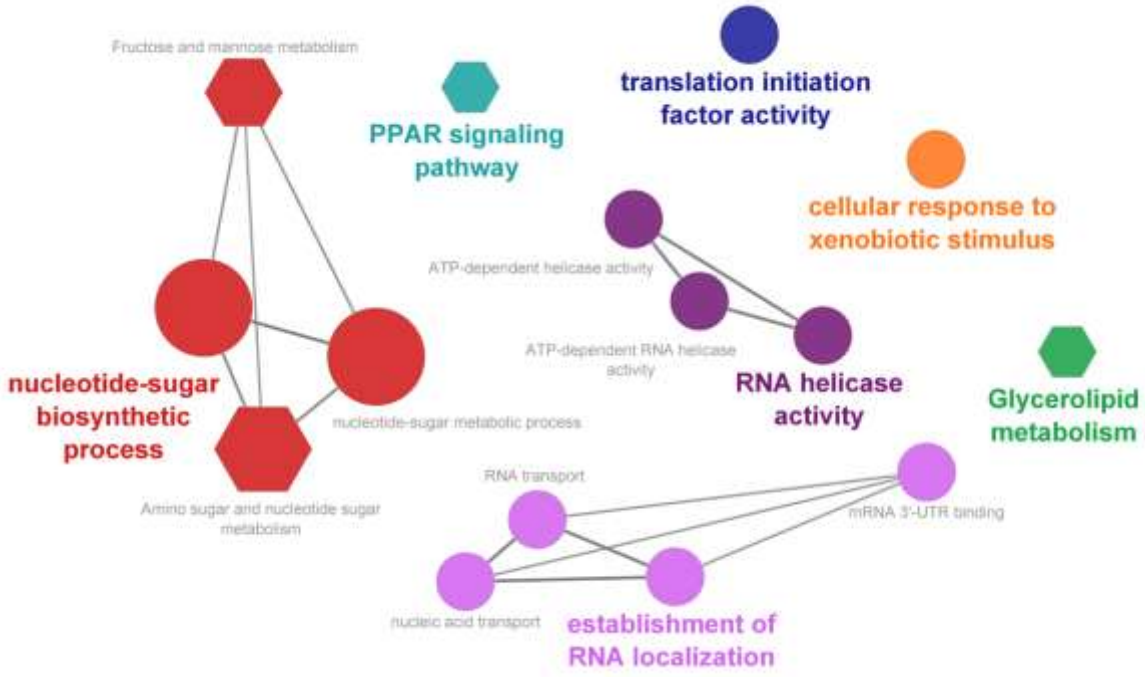

Figure 2.3. ClueGO visualization of proteomics data of functionally grouped networks of biological processes formed from significantly dysregulated pathways based on GO (circles) and KEGG (hexagons) databases. Larger nodes indicate greater significance. Pathways were built using at least three differentially abundant proteins ($p \leq 0.05$) in the liver tissue of male *P. promelas* after 96 hr exposure to the high (56.7 μgL^{-1} FLX) treatment.

2.3.6 *Brain and Liver Transcriptomics in Male* P. promelas

Following 96-hour FLX exposure, whole transcriptome sequencing revealed 28 differentially expressed genes (DEGs) in the brains of males in the medium treatment group and eight DEGs in the high treatment group, relative to the control (FDR-adjusted p-value≤0.05). In both the medium and high treatment groups, there was an equal proportion of up- and downregulated DEGs. No significant GO terms or KEGG pathways were identified in the enrichment analyses of either group. Between the medium and high groups, there were three common and highly upregulated DEGs: *dnmbp*, encoding for dynamin-binding protein; *socs5a*, encoding for suppressor of cytokine signaling; and *scamp4*, encoding for a secretory carrier-associated membrane protein-4 (Table 2.1; Figure E.3; Table E.5; Table E.6).

dnmbp is a multi-scaffold protein involved in GTPase activity and is highly concentrated at synapses and interacts with dynamin to regulate actin, the movement of synaptic vesicle pools, and endocytosis (Casoli et al., 2012). This function is linked to a reduced expression of the dopamine receptor (Beaulieu and Gainetdinov, 2011). A study by Cunha et al. (2018) demonstrated that FLX exposure in zebrafish resulted in a decrease in mRNA levels encoding for the dopamine receptor. Alteration in the dopaminergic system can negatively impact cognition, circadian rhythms, locomotor activity, reproduction, and behaviour in fish species (Beaulieu and Gainetdinov, 2011; Cunha et al., 2018; Fjorback et al., 2011; Mennigen et al., 2011). Notably, observed in rats, FLX was shown to increase extracellular dopamine in the hypothalamus (Koch et al., 2002). In some teleost species, seasonal control of reproduction is linked to increased serotonin and decreased dopamine levels (Mennigen et al., 2008). Prior to the present study, no published work has reported enrichment of *dnmbp* resulting from FLX exposure; however, given

it was highly upregulated (log2[fold-change]>19) in both FLX treatment groups, it may be an important early marker of exposure and should be further investigated.

Evidence that FLX may affect immune response was demonstrated in the significant upregulation (log2[fold-change]>21) of *socs5a* in both FLX treatment groups. The expression of *socs5a* is typically under strict regulation in fish (Wang et al., 2011). *socs5a* is induced by cytokines and acts in a negative-feedback loop to inhibit cytokine signal transduction. Reported in mice, serotonin is linked to the development of inflammatory processes by regulating cytokine secretion (Cloëz-Tayarani and Changeux, 2007). As FLX is known to increase the abundance of serotonin, one could infer that this, in turn, leads to an influx of cytokine production thereby triggering upregulation of *socs5a* expression.

scamp4 (log2[fold-change]>23), upregulated in both medium and high groups, is a member of a family of SCAMPs, which manage vesicles in various cell types throughout the body. More specifically, *scamp4* is a negative regulator of vesicle movement and over-expression could result in an increase in vesicular secretion (Krebs and Pfaff, 2001). Research by Krebs and Pfaff (2001) indicates that *scamp4* is associated with reproductive behaviour in the central nervous system of female rats. Aromatase, *cyp19a1a*, significantly over-expressed (log2[fold-change] = 3.1) in the brains of the high group, is also involved in reproductive functions and behaviour in teleosts (Hinfray et al., 2006; Lephart, 1996). Mennigen et al. (2010a) observed a similar over-expression of aromatase in the testes of adult male goldfish induced by FLX exposure. Contrary, Lister et al. (2009) found a decreased expression of aromatase in the ovaries of adult female zebrafish following FLX exposure. However, no previous research has observed differential expression of aromatase in the brain of fish. In the present study, these findings suggest that FLX can interfere with transcriptomic expression involved in reproductive function and behaviour, which could be

linked to the observed decrease in reproductive output (Section 2.3.3); however, further work is needed to elucidate the importance of these molecular signatures.

Table 2.1. Summary of the four key DEGs identified in the brains of FLX-treated male fathead minnows. Three of the DEGs are common between both treatment groups whereas aromatase was identified in the high group only.

Gene ID	**Description**	**Downstream Implications**	**log2(Fold-Change)**	
			Medium	**High**
dnmbp	Dynamin-binding protein	Cognition; circadian rhythms; locomotor activity; reproduction; behaviour	19.9	19.9
socs5a	Suppressor of cytokine signaling	Immune response & inflammation	21.6	22.5
scamp4	Secretory carrier-associated membrane protein-4	Reproductive function & behaviour; immune response	23.3	23.5
cyp19a1a	Aromatase	Reproductive function & behaviour		3.09

In the livers, transcriptomic analyses revealed 14 and 32 DEGs in the medium and high treatment groups, respectively (FDR-adjusted p-value≤0.05; Figure E.3; Table E.7; Table E.8). Of the 14 dysregulated genes in the medium treatment, five were downregulated and nine were upregulated. Among the 32 dysregulated genes in the high treatment, 21 were downregulated and 11 were upregulated. Between the medium and high treatments, there was one common DEG identified, *pla2g7*, encoding for platelet-activating factor acetylhydrolase, and which was downregulated in both treatments. *pla2g7* is an enzyme that specifically inactivates platelet-activating factors (PAFs), which are involved in inflammatory response and apoptosis (McIntyre et al., 2009). Interestingly, PAFs are mediators of persistent depression in coronary artery disease patients (Mazereeuw et al., 2013). Those authors suggested that SSRIs may act as anti-inflammatory agents and may interact with PAFs in the patients. The true role of *pla2g7* in terms of FLX exposure in fish has not yet been characterized; however, the research by Mazereeuw et al. (2013) does provide some possible context.

No significant GO terms or KEGG pathways were identified in the enrichment analysis of the DEGs in the liver tissues of fish in the medium group. Among the liver DEGs from the medium group, the most highly upregulated was *lipg*, which encodes for endothelial lipase, an enzyme that contributes to lipoprotein degradation and plays a major role in high-density lipoprotein cholesterol metabolism in vertebrates (Table E.7). Endothelial lipase is expressed in various tissue types, including the liver, where it is important for the maintenance of HDL cholesterol levels, structure, and metabolism (Holmes et al., 2011). A report by McCoy et al. (2002) showed that overexpression of endothelial lipase profoundly altered plasma lipoprotein levels in mice.

Enrichment analysis of the liver DEGs from the high group identified three GO terms and one KEGG pathway (Figure 2.4). The consensus was the dysregulation of biological processes

involved in the biosynthesis and metabolism of fatty acids. Whole transcriptome analysis by Wong et al. (2013) in male zebrafish treated with FLX also found fatty acid metabolism and biosynthesis-related GO terms over-enriched and attributed this outcome to a shift in macromolecule resources. Similarly in mice, FLX caused dysregulation in lipogenesis and lipolysis in hepatocytes, which led to increased lipid accumulation in the liver (Feng et al., 2012). In a metabolite enrichment analysis of zebrafish embryos exposed to FLX, significant metabolic phenotypes identified included sphingolipid metabolism, beta oxidation of fatty acids, and phospholipid biosynthesis (Huang et al., 2017). Exposure to aqueous FLX in zebrafish larvae resulted in dysregulation of several metabolic pathways including lipid metabolism, a response attributed to energy conservation (Craig et al., 2014). The findings of the present study are biologically relevant and may serve as an early marker of the increased appearance of lipid-type vacuolation in the liver (Section 2.3.4). Future investigations should further analyze the link between these molecular signatures and their potential histopathological implications.

Regarding enrichment analysis, no overlap was observed between the transcriptome and proteome; however, there were three common features between the respective lists of DEGs and DAPs in the high group (Figure 2.5). It is not unexpected to see differing responses at the transcriptome and proteome levels as they do not necessarily occur concurrently, especially if the response is compensatory or adaptive (Simmons et al., 2015). Reportedly, current literature suggests only a modest correlation between levels of mRNA and their corresponding proteins (Maier et al., 2009; Muers, 2011). This observation can be attributed to several mechanisms including post-transcriptional and -translational modifications, alternative promotors, alternative splicing, and epigenetic mechanisms, all of which can result in varying proteoforms originating from any given gene (Choudhuri, 2004; Kumar et al., 2016; Liu et al., 2016). In addition, some

cellular processes and molecular functions are more stable and tightly regulated than others, which could influence dysregulation in response to xenobiotic exposure. For example, pathways involved in transcription and translation are typically rather stable, whereas metabolic processes are generally more variable (Martyniuk and Houlahan, 2013). Regardless, while the pathways and terms identified from the proteomic analysis were not necessarily directly linked to lipid metabolism, they may have some influence on and from lipid-related processes. For example, dysregulation of lipid metabolism, as observed in the transcriptome, may be an upstream process to the enrichment of the PPAR signaling pathway. Among the DEGs, the most highly downregulated (log2[fold-change]=-2.25) was *scd*, encoding for stearoyl-CoA desaturase (delta-9-desaturase), an enzyme which functions in lipid metabolism (Pereira et al., 2003). Notably, stearyl-CoA desaturase was identified in fatty acid biosynthetic and metabolic processes in the transcriptome as well as the PPAR signaling pathway in the proteome.

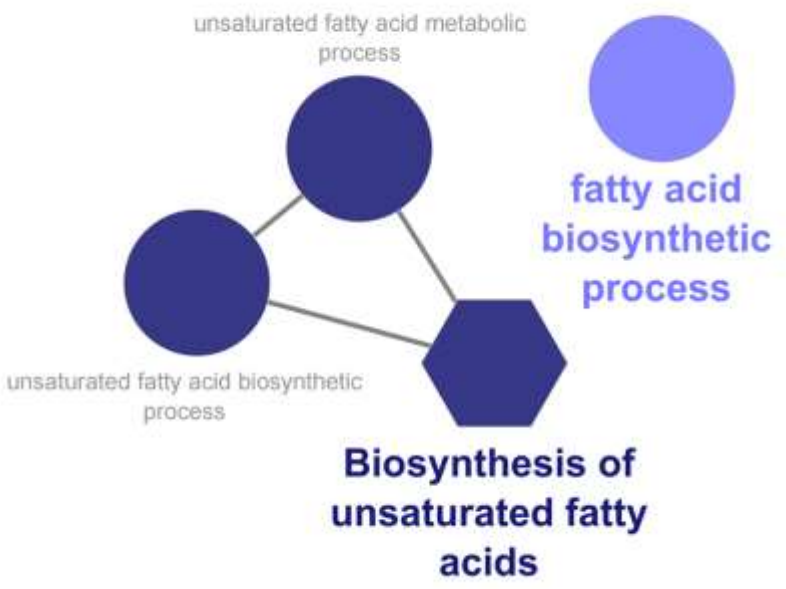

Figure 2.4. ClueGO visualization of transcriptomics data of functionally grouped networks of biological processes formed from significantly dysregulated pathways based on GO (circles) and KEGG (hexagon) databases. Larger nodes indicate greater significance. Pathways were built using at least two differentially expressed genes ($p \leq 0.05$) in the liver tissue of male *P. promelas* after 96 hr exposure to the high (56.7 μgL^{-1} FLX) treatment.

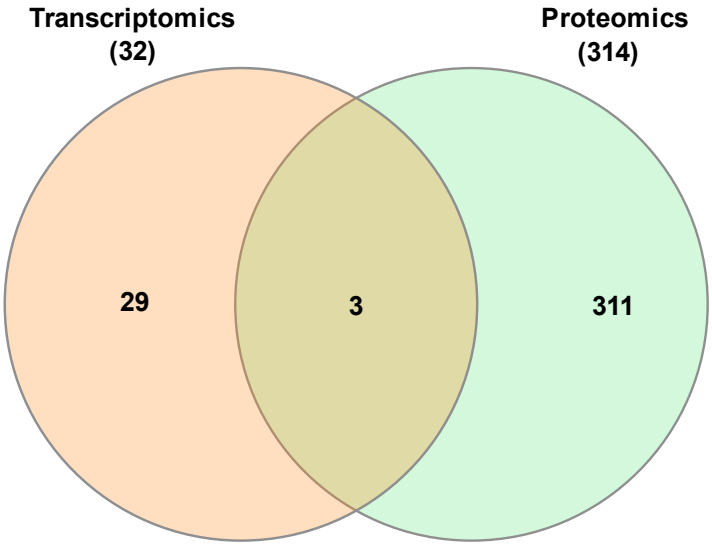

Figure 2.5. Intersection of DEGs (orange) and DAPs (green) in the liver of the high (56.7 µgL^{-1} FLX) treatment group. The abundance of three features was dysregulated at both the transcriptome and proteome levels. (Note: sizes of circles do not reflect the relative numbers of DEGs or DAPs in their respective groups. The Venn diagram was constructed using InteractiVenn (Heberle et al., 2015)).

2.4 Conclusion

The objective of the present research was to determine if molecular toxicity pathways identified through whole proteome and transcriptome analyses could help predict apical level responses following FLX exposure in adult fathead minnows. FLX exposure induced measurable responses in fecundity, liver histopathology, liver proteomics, and brain and liver transcriptomics. Pathway dysregulation of fatty acid-related processes indicates a change in energy resources of the exposed fish and is a molecular response that could lead to lipid-type vacuolation of hepatocytes, as observed in the histopathology analysis. Differential expression in the transcriptome was observed in the liver of the exposed fish and suggested dysregulation of pathways involved in the biosynthesis and metabolism of fatty acids. Additionally, dysregulation of PPAR signaling observed in the proteome of the liver could be a downstream effect of the dysregulation of fatty acid-related processes because fatty acids are endogenous ligands of PPARs.

Common dysregulated genes in the brain of both analyzed treatment groups were related to cellular signaling processes that are influenced by serotonin levels. Cellular signaling pathways including the mechanisms of *cyp19a1a* and *scamp4* may be related to reproductive behaviour, and therefore, could influence reproductive output, as observed in the present study. A significant decrease in spawning rate was measured in all FLX treatment groups following the 21-day exposure, which suggests that FLX may have potential impacts on the fitness of FHM at environmentally relevant concentrations. The entire adverse outcome pathway to this response has not yet been fully elucidated; however, the overexpression of behaviour-related genes in the brain of male fish is an important indicator. It is also possible that the enrichment of lipid-related processes is an indication of dysregulated energy metabolism, and therefore, a presumable decrease in energy allocated to reproductive behaviour and mechanisms. Reproduction is a

complicated biological process, and therefore, it is difficult to directly connect molecular responses to their possible downstream effects. In conclusion, this work demonstrated the potential of large-scale omics data to elucidate the complex physiological response of adult FHM to FLX, adding to the growing body of literature on the utility of NAMs in support of chemical hazard assessment.

CHAPTER 3: GENERAL DISCUSSION

3.1 Research Overview and Key Findings

The intention of this thesis research was to use FLX as the model compound for the development of a novel mechanism-based toxicity assay because of its well-defined adverse outcomes in fish species, yet a lack of existing data regarding its molecular toxicity pathways. The main objective of this thesis was to identify molecular signatures of FLX exposure that could be predictive of apical level effects in fathead minnows. This information would then provide much-needed data on responses across levels of biological organization, from the transcriptome and proteome to the whole animal. By characterizing these responses and assembling toxicity pathways, these molecular signatures can provide important context for often-researched endpoints of regulatory relevance and support environmental risk assessment. Therefore, the objectives of this study were to 1) determine if acute exposure to FLX alters molecular response patterns in *P. promelas* using whole proteome and transcriptome analyses, 2) characterize apical level effects (morphometry, fecundity, and histopathology) of regulatory relevance using a 21-day short-term reproductive assay (OECD, 2012), and 3) establish molecular toxicity pathways for FLX in *P. promelas* that can inform biological outcomes of regulatory relevance through integration of omics, histological and apical outcome data.

Exposure to FLX in adult fathead minnows induced measurable responses at the four levels of biological organization examined in this experiment. By analyzing the rate of embryos spawned per female over the duration of the 21-day exposure, it was revealed that all three tested levels of FLX resulted in significantly reduced fecundity. Notably, little deviation from the control slope in the treated groups was observed until the latter half of the exposure period, suggesting a considerable delay for the reduced fecundity response to emerge. SSRIs inhibit the serotonin

transporter within minutes of exposure; however, it takes several weeks of treatment to achieve their full effect – a phenomenon well-described in the therapeutic application in humans but not yet in endpoints, such as fecundity, in fish. Following the initial inhibition of the serotonin transporter there is an increase of synaptic serotonin. Thereafter, the serotonin autoreceptors mediate a negative feedback of subsequent neuronal serotonin release, which allows for changes in signal transduction and neuronal firing rate (McDonald, 2017).

Histopathological assessment was performed on the liver tissue of male fathead minnows exposed to the high treatment of FLX for 21 days. The assessment showed that although there were no overt changes to the tissue, there was a presence of lipid-type vacuolation in two of the five fish analyzed. There is little empirical evidence in current literature to suggest that fish with vacuolated livers as observed in our study would function abnormally (Wolf and Wheeler, 2018); however, this thesis research indicated altered molecular expression (e.g., PPAR signalling and lipid biosynthetic and metabolic processes) in the liver which may demand further investigation.

Whole proteome analysis of the liver of male fish exposed to the high treatment revealed enrichment of several pathways and terms of biological processes and molecular functions. In particular, the PPAR signaling pathway, of which serotonin metabolites may act as endogenous ligands, was significantly dysregulated in fish exposed to FLX. The pathway enrichment in grouped terms including RNA helicase activity, establishment of RNA localization, and translation initiation factor activity is a strong indication that there is dysregulation in transcription and translation caused by FLX. The enrichment of biosynthetic and metabolic processes of nucleotide sugars, upstream substrates of post-translational modification pathways, further indicates that FLX has an influence on gene expression (Hadley et al., 2014; Orellana et al., 2016).

Transcriptomic analysis revealed differentially expressed genes in male fish exposed to medium and high levels of FLX. Enriched terms identified from these dysregulated genes were involved in biosynthesis and metabolism of fatty acids. Pathway dysregulation of fatty acid-related processes may indicate a change in energy resources of the exposed fish and is a molecular response that could lead to lipid-type vacuolation of hepatocytes, as observed in the histology analysis. Additionally, fatty acids are endogenous ligands to PPARs as well, and therefore, this transcriptomic response may be upstream of the dysregulation observed in the proteome.

In the transcriptome of the brains, four key genes were highly overexpressed compared to the control. Two are known to be involved in reproductive pathways and behaviour: secretory carrier-associated membrane protein-4 (*scamp4*) and brain aromatase (*cyp19a1a*). These findings suggest that FLX may interfere with reproductive function and behaviour at the transcriptomic level. This response could be linked to the observed reduced fecundity; however, further work is needed to elucidate the importance of these molecular signatures in reproductive output. Also significantly upregulated, dynamin binding protein, *dnmbp*, a synaptic membrane protein which is potentially linked to the expression of the dopamine receptor (Beaulieu and Gainetdinov, 2011). Alteration in the dopaminergic system can negatively impact cognition, locomotor activity, and behaviour in fish (Cunha et al., 2018; Fjorback et al., 2011). The upregulation of suppressor of cytokine signalling (*socs5a*) demonstrates that FLX may affect immune and inflammatory response of fathead minnows.

Overall, these findings suggest some linkages between responses measured across biological levels of organization in this study. The observed pathway dysregulation of fatty acid-related processes in the liver transcriptome indicates a change in energy resources of the exposed fish and is a molecular response that could be related to lipid-type vacuolation of hepatocytes, as observed

in the histopathology analysis. Additionally, dysregulation of PPAR signaling observed in the proteome of the liver could be a downstream effect of the dysregulation of fatty acid-related processes because fatty acids are endogenous ligands of PPARs. Common dysregulated genes in the brain were related to cellular signaling processes that are influenced by serotonin levels. Pathways including the mechanisms of *cyp19a1a* and *scamp4* may be related to reproductive behaviour, and therefore, could influence reproductive output, also observed in this study. A significant decrease in spawning rate was measured in all treatment groups following the 21-day exposure, which suggests that FLX may have potential impacts on the fitness of fathead minnows at environmentally relevant concentrations. The entire toxicity pathway leading to this to this response has not yet been fully elucidated; however, the overexpression of reproductive behaviour-related genes in the brain of male fish is an important indicator. It is also possible that the enrichment of lipid-related processes is an indication of dysregulated energy metabolism, and therefore, a presumable decrease in energy allocated to reproductive behaviour and mechanisms. Reproduction is a complicated biological process, and therefore, it is difficult to directly connect molecular responses to their possible downstream effects.

3.2 Shortcomings of Research

Although this research does provide promising insights to the mechanistic toxicity of FLX, as well as the application of multi-omics in environmental risk assessment, there are still uncertainties and shortcomings that must be addressed. Fecundity was measured and revealed significant reproductive impairment in the FLX-exposed fathead minnows. Arguably, this would be considered a relevant measurement to the exposed female fish; however, molecular responses were measured in the male fish only. This shortcoming may influence the interpretation of the relationship between the transcriptomics and proteomics data and fecundity. It is certainly true

that differences of sex exist between male and female fish in reproductive function and the molecular responses that would result from exposure. For example, the hypothalamic-pituitary-gonadal (HPG) axis, which is responsible for the control of reproduction, is known to be influenced by serotonin on all levels (McDonald, 2017). For this reason, it would be practical to examine the equivalent transcriptomic and proteomic responses in the FLX-exposed female fish with the aim of gaining insight to the mechanism of action leading to reduced fecundity. However, measurements in the male fathead minnows are still relevant and important to consider as their reproductive behaviour is a significant driver of the reproductive success of a population. For example, the mating activities of the males initiates female spawning. Sebire et al. (2015) examined the effects of FLX on reproductive endpoints in three-spined sticklebacks following a 21-day exposure. Change in spawning was not measured directly, but the authors did observe a significant decrease in nest quality, a critical factor in stickleback reproduction, in groups exposed to aqueous FLX at comparable concentrations, indicating a challenge in attracting female partners (Sebire et al., 2015). Behaviour was not directly measured in this thesis research because it has been previously well-characterized in fish exposed to FLX (Gaworecki and Klaine, 2008; Painter et al., 2009; Weinberger II and Klaper, 2014). Qualitative observations were made throughout the duration of the experiment such as reduced aggression and swimming with close association to the water surface in the fish in the FLX-spiked tanks, similar to outcomes reported by Gaworecki and Klaine (2008) and Theodoridi et al. (2017). These behavioural changes could potentially have consequences in reproduction. Therefore, it is reasonable to assume that behaviour-related molecular pathways are an early indication of change in reproductive output.

Comparison of the transcriptomic and proteomic signatures was performed qualitatively because few signatures could be identified. Only three features were common between the lists of

differentially expressed genes and proteins at the high FLX treatment level; therefore, a correlation analysis was not practical. Moreover, there were no similarities between the terms and pathways of the transcriptome and proteome identified in the ClueGO enrichment analysis. Assessment of these analyses indicated a relationship between biosynthetic and metabolic processes of fatty acids at the transcriptome and PPAR signalling at the proteome; however, few other linkages could be derived. It is not unexpected to see differing responses at the transcriptome and proteome levels as they do not necessarily occur concurrently (Simmons et al., 2015). In addition, some cellular processes and molecular functions are less variable and tightly regulated than others, which could influence enrichment of molecular signatures in response to xenobiotic exposure. For example, terms involved in transcription and translation are typically rather stable, whereas metabolic processes are generally more variable (Martyniuk and Houlahan, 2013). Regardless, while the pathways and terms identified from the proteomic analysis were not necessarily directly linked to lipid metabolism, they may have some influence on and from lipid-related processes. The proteome can be considered more complex than the transcriptome when accounting for post-translational modifications and varying isoforms and therefore provides a different perspective and can enhance and supplement interpretation of molecular outcomes (Garcia-Reyero and Perkins, 2011).

Due to practical restraints of the sample excision procedure, the entire brain tissue of each male fish was sampled for transcriptomic analysis rather than specific regions. This raises the concern that there may be diminishment of the transcriptomic responses that are only active in specific regions of the brain where serotonergic neurons are more concentrated (e.g., hypothalamus; Lillesaar, 2011). This may provide explanation why we observed so few differentially expressed genes in both medium and high FLX treatments.

3.3 Future Directions

The results of this research increase the scope of applications in which multi-omics can be used to identify molecular toxicity pathways predictive of apical outcomes. The investigations also successfully identified key molecular features of differentially enriched toxicity pathways relating to FLX exposure in fathead minnows. However, additional research would enhance the knowledgebase of multi-omics in ecotoxicology as well as the effects of FLX on the fathead minnow. Notably, analysis of the metabolome would be beneficial in validating the observations made in the transcriptome and proteome. Metabolomics is the global analysis of endogenous small molecule metabolites and their relative abundance in a tissue sample, and therefore, is regarded as a comprehensive evaluation of biological responses/activity at a given time (Huang et al., 2016; Villeneuve and Garcia-Reyero, 2011). Therefore, utilizing this analysis of the liver could further elucidate the fatty acid metabolic processes that were enriched in the transcriptome. This data could help improve our understanding of the mechanism of action of FLX in FHM.

Future research should examine the transcriptomic and proteomics responses in female fathead minnows, given they have key physiological differences that may influence how FLX affects molecular responses such as endocrine signalling (e.g., HPG axis) (Dorelle et al., 2017; McDonald, 2017; Mennigen et al., 2017). Features identified in males that are related to reproductive function may also be prominent in females and therefore could be extrapolated to the decreased fecundity observed in this study. Alternatively, there could be an abundance of differentially expressed genes that have not yet been identified in current published work. For example, aromatase was highly overexpressed in the brain transcriptome of male FHM exposed to the high FLX treatment. Reportedly, male fish have shown overall higher brain aromatase activity than females (Diotel et al., 2010). Reduced fecundity was found to be a key outcome of FLX

exposure in fathead minnows at all tested levels of FLX; therefore, broadening our understanding of the mechanistic events that lead to this outcome is critical for our understanding of its toxicity. The enrichment of brain aromatase from FLX exposure in fish is a novel observation therefore, measuring transcriptomic and proteomic responses in female FHM when exposed to FLX could aid in closing our current knowledge gaps.

Many differentially expressed features identified across the transcriptome and proteome assessed in this study have not been well-studied in fathead minnows in response to FLX exposure. Therefore, interpretation was drawn from extrapolation of other vertebrate models in the scientific literature as well as form inferences on their relationship to the serotonergic system or known physiological effects of FLX. Further research targeting these features may be very helpful in elucidating the mechanistic action of FLX in the fathead minnow. For example, *dnmbp* was highly enriched (log2[fold-change]>19) in both medium and high FLX-treated fish brains; however, its relation to FLX toxicity is still uncertain. Its function may be associated with the dopaminergic system, which may, in turn, relate to the serotonergic system. Further investigation should examine the mechanism of this specific gene in association with serotonin and FLX toxicity in fish. All considered, the molecular signatures found in both the liver and brain provided key insights to downstream effects of FLX exposure and can be used for calibration of future studies of this kind. These recommendations of future research would validate the work reported in this thesis by supporting the uncertainties addressed above. Expanding our knowledge of the mechanism of toxicity of FLX will exemplify how these new approach methods can be applied in novel contaminants of concern.

3.4 Concluding Statement

This thesis research was conducted to gain a better understanding of the effects of FLX exposure on fathead minnows and to provide novel data on responses across levels of biological organization, from molecular to whole animal responses. The specific objectives were to identify key molecular signatures of FLX exposure using whole transcriptomics and proteomics analyses as well as characterization of apical level outcomes including alterations of histopathology and fecundity. The results indicate that exposure to FLX can induce responses across a range of physiological parameters at all levels of biological organization and suggest that multi-omics investigations have the potential to be used for predicting adverse outcomes. In addition, this thesis work contributed novel information on the molecular signatures in fish indicative of FLX exposure never previously reported in fathead minnows. These findings add to the growing body of evidence regarding the potential links between molecular mechanistic and key physiological events of FLX toxicity to adverse outcomes of regulatory relevance.

The research conducted in Chapter 2 of this thesis was part of a large, collaborative research project, the EcoToxChip project (EcoToxChip.ca), that aims to develop and validate quantitative PCR arrays and a data evaluation tool (EcoToxXplorer.ca) for the characterization, prioritization, and management of environmental contaminants in model laboratory species and native species of concern (Basu et al., 2019). Specifically, the project aims to link molecular signatures (e.g., transcriptomics, proteomics) to apical outcomes of exposure to eight model compounds in fish, amphibian, and avian species. The findings of this study informed the development of the fathead minnow EcoToxChip by generating information critical to support gene selection for the qPCR array. The overall goal of this project is to support current risk assessment and chemical management programs by applying new approach methods, such as performed in this thesis

research, to help reduce the need for traditional testing methods (e.g., live animal tests to assess survivability or other fitness-related endpoints), and therefore, improve regulatory decision making while minimizing the need for time-consuming, expensive, and ethically questionable live animal tests. The research of this thesis can help bridge the transition to utilizing contemporary scientific knowledge in mechanistic toxicology in these decisions because it demonstrated the potential of predicting adverse outcomes using a mechanistic approach.

This research found that the responses observed at the transcriptome and proteome level, especially the identification of novel signatures and pathways, have the potential to drive the direction of future research into characterizing the underlying mechanisms of toxicity in fish species. A mechanistic approach is beneficial in toxicological research in the assessment of new environmental contaminants of concern because it can help elucidate the molecular events that take place and reveal new facets of biological and cellular function while improving the efficiency, economy, and reliability of data collection (Basu et al., 2019; Villeneuve and Garcia-Reyero, 2011). Because of the dynamics of gene expression, it can be difficult to interpret transcriptomic data regarding predictivity of adverse outcomes. Integration with analyses such as proteomics could address these uncertainties, and potentially help in building toxicity pathways. For example, as demonstrated in Chapter 2, enrichment of PPAR signalling pathway solidified the importance of fatty acid-related processes, and therefore their downstream implications in lipid-type vacuolation as seen in liver histopathology. Moreover, the enrichment of transcription- and translation-related processes evidently signifies FLX does influence gene expression.

By identifying sensitive and more specific endpoints, future toxicity research can be directed in more meaningful ways. Some antiquated apical endpoints (e.g., morphometric measurements) are not as sensitive to xenobiotic exposure or yield inconsistent results. They also

require long-term exposures and many animal lives which are costly to maintain. A mechanistic approach, as demonstrated in the present study, allows researchers to examine the biomarkers of exposure, before adverse effects take place, and therefore, address these critical concerns in traditional testing programs. By understanding a specific molecular key event that initiates a toxicity response, one can then infer any other chemical compound with a similar mechanism would result in the same outcome. In conclusion, this work achieved our objectives in demonstrating the potential of large-scale omics data to elucidate the complex physiological response of adult FHM to FLX as well as added to the growing body of literature on the utility of these methods in support of chemical hazard assessment.

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APPENDICES

Appendix A: FLX Exposure Experiment

Dissolved oxygen was maintained at >80% by aeration stones. Each aquarium contained two breeding tiles (halved sections of PVC pipe) used as substrate for laying embryos on as well as for environmental enrichment. The fish were fed *ad libitum* three times daily with thawed *Chironimidae* larvae (Hikari Sales Inc., Haywards, USA). Excess food particles and waste were removed daily. Any minnows that did not reproduce during the acclimation period or showed signs of stress or impaired health were replaced. Sex was determined by examination of secondary sex characteristics including male banding and nuptial tubercles (OECD, 2012). The photoperiod was maintained at 16 hours of light and 8 hours of dark with illumination measured between 800-1000 lux. Upon exposure initiation, behaviour, health, and mortalities were recorded daily, and dead fish were removed. Tissues were fixed in CalEx-II (Fisher Scientific; CAT# CS511-4D) for 48 hours, and then stored in 70% ethanol until processing. Liver and brain of the remaining 21-day fish were excised, weighed, and flash-frozen in liquid nitrogen before being stored at -80°C for future analyses. Observations of abnormalities were also recorded for each fish.

Appendix B: Analysis of FLX Exposure Concentrations

Analysis of FLX concentration was conducted at SGS AXYS Analytical Services Ltd (Sidney, BC, Canada). Reference FLX was obtained from Millipore Sigma (Burlington, MA, USA) and d_5-FLX was obtained from CDN Isotopes (Pointe-Claire, QB, Canada). FLX was measured following the method described by Long et al (2013), with minor revisions. FLX samples were extracted and analyzed by solid phase extraction/isotope-dilution LC-MS/MS. Based on the expected exposure concentrations, a sub-sample was spiked with d5-FLX, diluted to 500 mL in reagent water, and buffered to a pH of 3.8 using a sodium acetate: acetic acid buffer.

Samples were then loaded on a 1g Oasis-HLB cartridge (Waters, Milford, MA, USA) and eluted using 12 mL of methanol and 6 ml of 1:1 acetone:methanol. Sample extracts were evaporated to dryness under a stream of N2 and reconstituted in 2 mL of solution of methanol with 0.1% formic acid. FLX concentration levels were measured using a Waters 2690 HPLC, equipped with Xterra MS C18 (Waters; 10.0 cm, 2.1 mm i.d., 3.5 µm particle size), coupled with Micromass Quattro Ultima MS/MS (Waters) operating in positive ion electrospray mode at unit resolution. Transitions used for FLX and d5-FLX were 310⟶148 and 315⟶153, respectively. The flow rate varied from 0.15-0.3 mL per minute. The column was maintained at 40°C. A gradient elution using mobile phases 0.1% formic acid buffer (A) and 1:1 acetonitrile:methanol (B) was used. The LC gradient program was as follows (time, %A): 0.0, 95%, 4.0, 95%, 22.5, 12%, 23.0, 0%, 26.0, 0%, 26.5, 95%, and 33.0, 95%. Source temperature was 120°C and desolvation temperature was 350°C. A seven-point calibration curve ranging from 0.375-1250 ng mL^{-1} was used to quantitate using 1/x weighted linear calibration and response relative to d5-Fluoxetine. Concentrations produced were thus recovery corrected.

Appendix C: Transcriptomics

Transcriptomic analysis was conducted on liver and brain tissues excised from male fathead minnows from the facility water control and medium, and high FLX treatment groups. Total RNA was extracted from tissue samples of individual fish using the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) in a QiaCube instrument (Qiagen). Pooled samples with an RNA Integrity Number (RIN) ≥ 9 were used for mRNA sequencing. Samples were diluted using sterile RNAse-free water to a final volume of 25 µL and concentration of 100 ng $µL^{-1}$ RNA. The RNA samples were pooled to yield one sample per replicate unit (n = 5 per treatment group) and stored at -80°C until sequencing.

Transcriptome sequencing was performed at the Genome Quebec Innovation Centre (McGill University, Montreal, QC, Canada). Steps for generating libraries from total RNA included the following: mRNA enrichment was performed using the NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs, Ipswich, MA, USA); cDNA synthesis was done using NEBNext RNA First Strand Synthesis and NEBNext Ultra Directional RNA Second Strand Synthesis Modules (New England BioLabs). Subsequent steps of library preparation were done using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs). Adapters and PCR primers were purchased from New England BioLabs. Libraries were quantified using the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems, Wilmington, MA, USA). Determination of average fragment size was done with a LabChip GX (PerkinElmer, Waltham, MA, USA) instrument. Once libraries were normalized and pooled, they denatured in 0.02N NaOH and neutralized using HT1 buffer. The pool was loaded at 225pM on an Illumina NovaSeq (Illumina, San Diego, CA, USA) S4 lane using Xp protocol. The run was performed for 2x100 cycles (paired-end mode). A phiX library was used as a control and mixed with libraries at 1% level. Base calling was performed with RTA v3.4.4. Program bcl2fastq2 v2.20 was applied to demultiplex samples and generate paired-end fastq reads.

Raw RNA-Seq fastq files were assessed using the EcoToxXplorer Galaxy server (https://galaxy.ecotoxxplorer.ca/) and were trimmed to a minimum phred score of 20 and a minimum sequence length of 35 bases per paired read using Trim Galore! (Krueger, n.d.). Kallisto quant (Bray et al., 2016) was used to align reads to the latest *P. promelas* reference genome build (NCBI Acc# GCA_016745375.1; Martinson et al., n.d.) and quantify abundances of RNA-Seq transcripts. Raw counts were filtered to remove gene features with less than 20 counts per million in at least five samples. Transcript count data was normalized by use of the trimmed mean of M

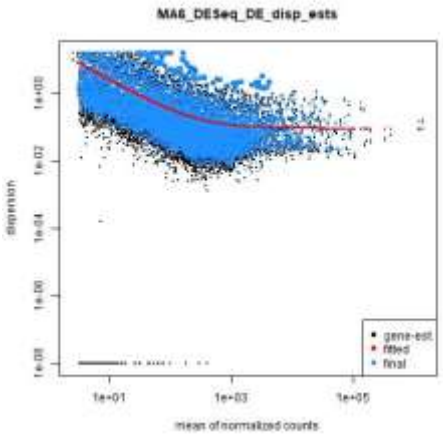
values approach (Robinson and Oshlack, 2010). Differential expression analysis was performed by utilizing R package DESeq2 (Love et al., 2014). Genes were considered significantly differentially expressed at a cut-off false discovery rate (FDR; Benjamini-Hochberg, BH) of ≤ 0.05 relative to the water control group.

Appendix D: Fecundity

Raw count data were organized as cumulative embryos spawned per surviving female per day. Evaluation determined the data did not align to a normal distribution; therefore, a repeated-measures design ANOVA was not an appropriate statistical test. Also noteworthy, the experimental unit from which data were collected was the tank of fish, as opposed to an individual, a violation of an assumption for a repeated-measures ANOVA test. Thorough evaluation of distribution uncovered that the data aligned best with a type I negative binomial distribution to account for zero inflation of the data. To find the best fit model of the data, four parameters (QQ plot, residual plot, dispersion, and zero inflation) that indicated were examined a strong fit using the DHARMa R package (Hartig, 2020), a tool for interpreting residuals of generalized linear mixed models. The investigation found that the best fit model for the data was a generalized linear mixed model analyzing the main effect of FLX treatment and time (days) as the covariate with a random error structure to account for the stratification of ranked aquaria across replicates. The model displayed acceptable homogeneity of variance, normality, and leverage of residuals. Analysis of the fecundity model revealed there was a significant interaction between FLX treatment and time; therefore, differences in trendlines of the rate of embryo production per surviving female were examined to draw the conclusion of FLX-induced effects on fecundity.

Appendix E: Figures and Tables

A



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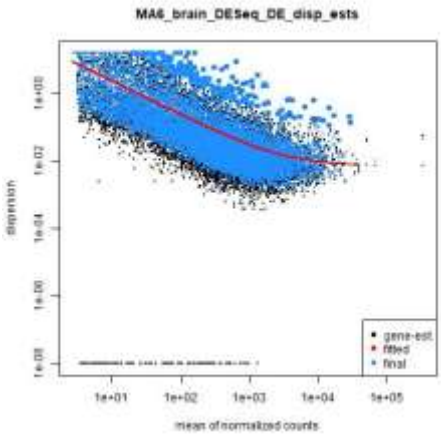

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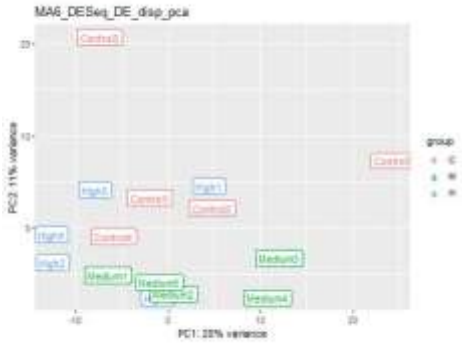

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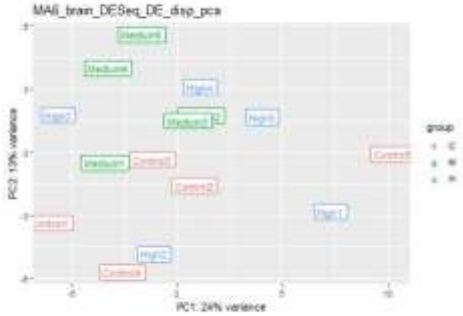
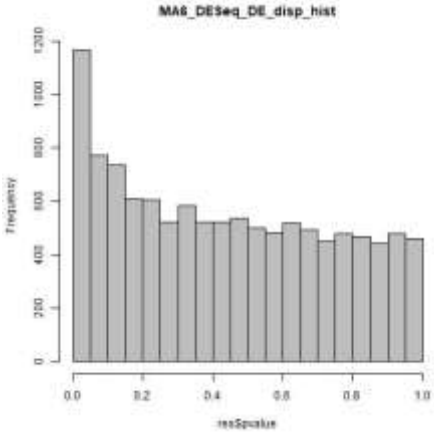


E



F

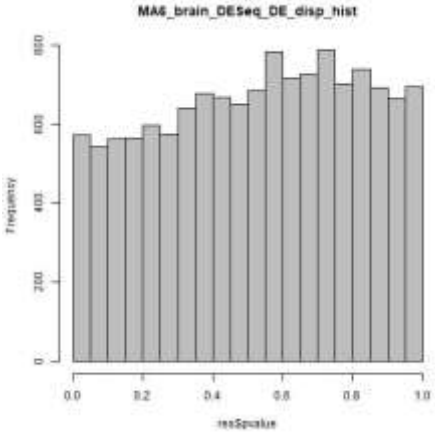


Figure E.1. Statistical graphics generated in R depicting dispersion (A, B), principal component analyses (C, D), and histograms (E, F) of the gene count data from liver and brain tissues, respectively.

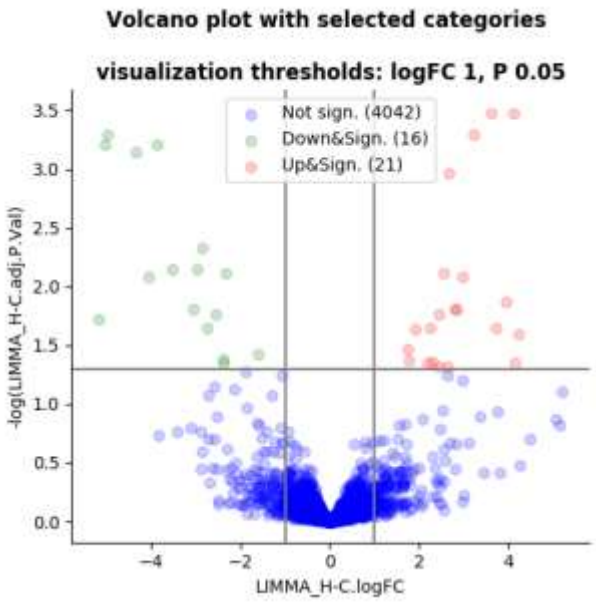


Figure E.2. Volcano plot depicting log(adjusted p-value) versus log(fold change) of differentially abundant proteins from the proteomic analysis of high FLX-exposed liver.

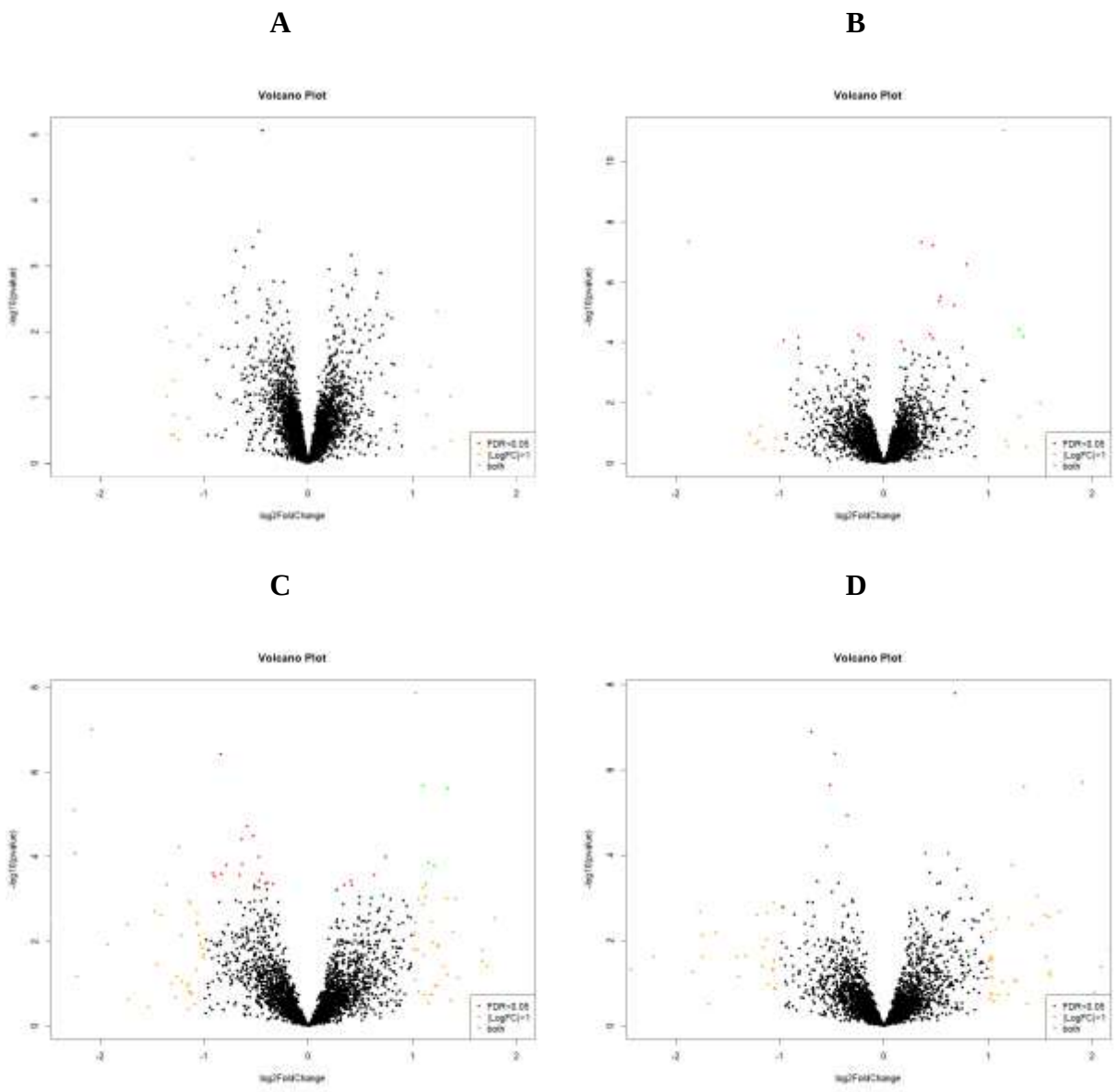


Figure E.3. Volcano plots depicting log(p-value) versus log2(fold change) of differentially expressed genes from the transcriptomic analysis of (A) high FLX-exposed brain, (B) medium FLX-exposed brain, (C) high FLX-exposed liver, and (D) medium FLX-exposed liver.

Table E.1. Water quality parameters (mean±SEM) over the exposure period. Parameters were measured daily from eight randomly selected aquaria over the 21 days of exposure.

		FLX Treatment		
	Facility Water	**Low**	**Medium**	**High**
Temperature (°C)	25.69±0.13	25.71±0.11	25.7±0.15	25.52±0.14
Dissolved Oxygen (%)	90.04±1.92	88.15±2.05	93.18±1.57	90.8±2.33
Conductivity (µS/cm)	493.35±4.04	480.38±13.28	493.6±3.67	495.63±3.44
pH	7.93±0.02	8.01±0.05	8.12±0.01	8.08±0.04
Ammonia (ppm)	0.28±0.05	0.34±0.07	0.23±0.05	0.23±0.03
Nitrate (ppm)	8.5 ± 1.62	7.8 ± 1.51	7.9 ± 1.68	7.9 ± 1.46
Nitrite (ppm)	0.46 ± 0.12	0.50 ± 0.14	0.55 ± 0.19	0.55 ± 0.16
Hardness (ppm)	167.86±7.00	175.86±2.02	176.5±2.50	178.17±2.03
Alkalinity (mg/L)	113.42±2.37	113.86±2.08	112.75±2.50	117.17±2.17

Table E.2. Nominal and actual concentrations of FLX throughout the 21-day exposure period as measured by LC-MS/MS.

		FLX Concentration (μgL^{-1})			
	Exposure Day (D)	**Control**	**7**	**28**	**112**
	D1	0.023	2.61	10.4	56.2
	D6	0.018	2.70	11.0	49.3
	D12	0.017	3.06	12.9	63.8
	D21	0.008	1.29	8.32	57.4
Mean ± SEM		0.017±0.003	2.42±0.39	10.7±0.94	56.7±3.0
Percent of Nominal			35%	38%	51%

D1 = Exposure Day 1; D6 = Exposure Day 6; D12 = Exposure Day 12; D21 = Exposure Day 21

Table E.3. Morphometric parameters (mean±SEM) including condition factor (K), hepatosomatic index (HSI), and wet body weight of adult *P. promelas* following 21 days of aqueous FLX exposure.

Treatment	Condition Factor (K)		HSI		Wet Weight (g)	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Facility Water	1.96±0.15	1.78±0.07	2.07±0.17	1.74±0.09	3.35±0.25	1.75±0.12
Low	2.03±0.15	1.90±0.12	1.80±0.22	2.28±0.30	3.93±0.32	1.81±0.15
Medium	2.23±0.19	1.79±0.07	2.01±0.18	1.62±0.53	3.23±0.30	1.70±0.12
High	2.12±0.14	1.85±0.13	2.03±0.31	1.70±0.12	3.32±0.31	1.56±0.09

Table E.4. List of GO terms that were identified in the ClueGO enrichment analysis of the DAPs in the liver of male *P. promelas* exposed to 56.7 μgL^{-1} for 96hr. Group p-value was Bonferroni-corrected. Arrows of associated genes indicate up- or down-regulation.

GO Term	**GO ID**	**Associated Proteins**	**Group p-value**
Cellular response to xenobiotic stimulus	GO:0071466	*F1Q8C3* (↓), *Q9DDU5* (↓), *B0UXS0* (↑), *Q7T2V2* (↓)	1.0E-2
Glycerolipid metabolism	KEGG:00561	*Q6DG38* (↑), *Q7ZWC2* (↑), *F1R0E7* (↓)	1.4E-2
PPAR signaling pathway	KEGG:03320	*Q6IMW5* (↓), *Q9DEX7* (↓), *F1QG70* (↓)	8.4E-3
Translation initiation factor activity	GO:0003743	*Q7SXU0* (↓), *Q7ZU67* (↑), *B8A6A1* (↑)	1.7E-2
RNA helicase activity	GO:0003724	*Q7ZU67* (↑), *E7FAV9* (↓), *F1R881* (↑)	2.0E-2
ATP-dependent RNA helicase activity	GO:0004004	*Q7ZU67* (↑), *E7FAV9* (↓), *F1R881* (↑)	2.0E-2
ATP-dependent helicase activity	GO:0008026	*Q7ZU67* (↑), *E7FAV9* (↓), *F1R881* (↑)	2.0E-2
Fructose and mannose metabolism	KEGG:00051	*Q6GMK8* (↑), *Q3ZB95* (↑), *Q6P951* (↓), *Q5SPD5* (↑)	1.4E-4

Amino sugar and nucleotide sugar metabolism	KEGG:00520	*Q6GMK8* (↑), *Q3ZB95* (↑), *Q6NYR8* (↑), *Q5SPD5* (↑), *Q7ZWD4* (↓)	1.4E-4
Nucleotide-sugar metabolic process	GO:0009225	*Q6GMK8* (↑), *Q3ZB95* (↑), *Q5SPD5* (↑), *Q7ZWD4* (↓)	1.4E-4
Nucleotide-sugar biosynthetic process	GO:0009226	*Q6GMK8* (↑), *Q3ZB95* (↑), *Q5SPD5* (↑), *Q7ZWD4* (↓)	1.4E-4
Establishment of RNA localization	GO:0051236	*Q08CK7* (↑), *Q9PW80* (↑), *E7FBU7* (↓)	2.1E-2
RNA transport	GO:0050658	*Q08CK7* (↑), *Q9PW80* (↑), *E7FBU7* (↓)	2.1E-2
mRNA 3'-UTR binding	GO:0003730	*Q08CK7* (↑), *Q9PW80* (↑), *E7F112* (↓)	2.1E-2
Nucleic acid transport	GO:0050657	*Q08CK7* (↑), *Q9PW80* (↑), *E7FBU7* (↓)	2.1E-2

Table E.5. Differentially expressed genes identified through whole transcriptomics in brains of male *P. promelas* exposed to 10.7 μgL^{-1} for 96hr (FDR-adjusted p-value ≤ 0.05).

Gene ID	**log2(FoldChange)**	**Description**
NP_001293024.1	-25.20091989	Probable ATP-dependent RNA helicase DDX58
F1QJB6	-23.73902458	ATM serine/threonine kinase
E7FAV9	-9.627110224	Mov10 RISC complex RNA helicase a
E7FG92	-9.030929801	Transmembrane and tetratricopeptide repeat-containing gene 2
E7F3S5	-4.436471797	Cerebellin gene 1
XP_021334158.1	-3.880901573	Voltage-dependent L-type calcium channel subunit alpha-1D gene 3
Q6JIY4	-1.701371474	Homeobox protein Hox-C13b
Q6DGB6	-1.407150268	Coiled-coil domain-containing protein gene 12
A0A286Y9S6	-0.777395494	ATP-binding cassette, sub-family D (ALD), member 3b
XP_021324196.1	-0.729410947	General transcription factor IIH subunit gene 1
XP_005174312.1	-0.636908889	Deformed epidermal autoregulatory factor-like
Q7T012	-0.576893266	Haloacid dehalogenase-like hydrolase domain-containing protein gene 2
F1QNX7	-0.237708437	UPF3B, regulator of nonsense mediated mRNA decay
F1R8R5	-0.161260346	Zinc finger protein gene 29
XP_698635.3	0.365280781	ATP-dependent 6-phosphofructokinase, liver type

F1QWD1	0.435525572	TatD DNase domain containing
Q7SYB8	0.473326913	Glycogen [starch] synthase gene 2
NP_001108032.1	0.538652387	Synaptic vesicle membrane protein VAT-1 homolog-like
D2CLZ9	1.197439913	Protein atonal-like
XP_017212581.1	1.301628033	Collagen alpha-6(IV) chain
XP_691507.2	1.977334364	E3 ubiquitin-protein ligase TRIM21
NP_001070859.1	2.729214066	Proto-oncogene serine/threonine-protein kinase pim-1
XP_699490.4	3.137644722	Kelch-like gene 6
XP_005157961.1	6.486633085	Myocardin-like
F1RCC9	7.701026723	Synaptopodin-like gene 2
E7F1U2	19.94757971	Dynamin-binding protein
B0EVP7	21.64251038	Suppressor of cytokine signaling gene 2
Q4V8U2	23.30958189	Secretory carrier-associated membrane protein gene 3

Table E.6. Differentially expressed genes identified through whole transcriptomics in brains of male *P. promelas* exposed to 56.7 μgL^{-1} for 96hr (FDR-adjusted p-value ≤ 0.05).

Gene ID	**log2(FoldChange)**	**Description**
XP_009292164.1	-21.42860602	Zinc/cadmium resistance protein-like
F1QHI5	-21.20080752	Intersectin 1 (SH3 domain protein)
Q6JIY4	-1.918082193	Homeobox protein Hox-C13b
NP_001292523.1	-0.442153067	EF-hand domain-containing protein D1
O42145	3.093676584	Aromatase
E7F1U2	19.92620667	Dynamin-binding protein
B0EVP7	22.54310792	Suppressor of cytokine signaling gene 2
Q4V8U2	23.47800229	Secretory carrier-associated membrane protein gene 3

Table E.7. Differentially expressed genes identified through whole transcriptomics in livers of male *P. promelas* exposed to 10.7 μgL^{-1} for 96hr (FDR-adjusted p-value ≤ 0.05).

Gene ID	**log2(FoldChange)**	**Description**
F1RCJ1	-0.69482	Serine/threonine/tyrosine-interacting protein
XP_017212026.1	-0.54872	Protein CutA-like
Q5RHM0	-0.51577	Platelet-activating factor acetylhydrolase
Q7T3A5	-0.4694	Ubiquitin-like gene 3
NP_001017866.2	-0.35204	Ubiquitin-like protein fubi and ribosomal protein S30
XP_001343838.1	0.398141	Serine/threonine-protein kinase TAO1 gene 1
Q7SYB8	0.617917	Glycogen [starch] synthase gene 2
NP_001316793.1	0.683473	Receptor-type tyrosine-protein phosphatase gamma gene 2
Q6P6E8	1.231859	ADP-ribosylation factor-like 5C
F1Q8X6	1.3435	Pyruvate dehydrogenase kinase, isozyme 2b
Q6DHQ3	1.907421	N-acyl-aromatic-L-amino acid amidohydrolase (carboxylate-forming) B
XP_022540357.1	2.253524	Inactive tyrosine-protein kinase-like
Q66I24	2.280388	Argininosuccinate synthase
F1QET5	4.088779	Lipase, endothelial

Table E.8. Differentially expressed genes identified through whole transcriptomics in livers of male *P. promelas* exposed to 56.7 μgL^{-1} for 96hr (FDR-adjusted p-value ≤ 0.05).

Gene ID	log2(FoldChange)	Description
F1QG70	-2.25129793	Stearoyl-CoA desaturase (delta-9-desaturase)
Q4VBT1	-2.24091598	Fatty acid binding protein 1-B.1
XP_021330511.1	-2.07749834	Interferon-induced very large GTPase-like gene 30
Q6PBI2	-1.35890929	Pancreatic progenitor cell differentiation and proliferation factor B
XP_009295418.2	-1.23955628	Atrial natriuretic peptide-converting enzyme
Q561X1	-0.91862366	Anaphase-promoting complex subunit gene 2
NP_001004577.1	-0.89811696	Bifunctional coenzyme A synthase
XP_005169882.1	-0.84171125	Glucosamine 6-phosphate N-acetyltransferase
B3DGL5	-0.83336706	Neurofibromin 2a (merlin)
F1R312	-0.7826869	Potassium channel, subfamily K gene 5
NP_001153567.1	-0.66145483	Phospholipase D1 gene 1
Q1LX18	-0.63656073	SHC (Src homology 2 domain-containing)-transforming protein gene 2
NP_001008628.1	-0.63203408	Serine/threonine-protein kinase PINK1, mitochondrial
Q5RHM0	-0.58511665	Platelet-activating factor acetylhydrolase
Q08C81	-0.52688609	Mediator of RNA polymerase II transcription subunit 19-A
NP_001292513.1	-0.47254809	Enoyl-CoA delta isomerase 1, mitochondrial
A0A140LFW7	-0.46890783	Rho guanine nucleotide exchange factor (GEF) gene 7

Q6PHG4	-0.44258391	Lipoyl synthase, mitochondrial
Q502E4	-0.41263587	NADH dehydrogenase (ubiquinone) Fe-S protein 4, (NADH-coenzyme Q reductase)
Q1MTD3	-0.39234957	mRNA cap guanine-N7 methyltransferase
XP_001920107.4	-0.33883639	Dedicator of cytokinesis protein gene 1
Q92005	0.34861625	Elongation factor 1-alpha gene 3
NP_958895.1	0.40973083	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial
XP_009289452.1	0.41883293	Phospholipid-transporting ATPase IG
NP_956748.1	0.63634897	Protein tyrosine phosphatase, non-receptor type 11, b
NP_001032658.1	0.73830065	Type I inositol 1,4,5-trisphosphate 5-phosphatase gene 1
XP_005161213.1	1.03370177	Baculoviral IAP repeat-containing protein gene 3
A0A0N4SU63	1.10395304	Complement component c3b, tandem
Q8AX86	1.12970466	Elongation of very long chain fatty acids protein gene 8
NP_001082978.1	1.15652865	D-beta-hydroxybutyrate dehydrogenase, mitochondrial
F1Q5S2	1.2094276	Protein Z, vitamin K-dependent plasma glycoprotein a
A0A0G2KHA1	1.33778377	Vinculin b

Table E.9. List of GO terms that were identified in the ClueGO enrichment analysis of the DEGs in the liver of male *P. promelas* exposed to 56.7 μgL^{-1} for 96hr. Group p-value was Bonferroni-corrected. Arrows of associated genes indicate up- or down-regulation.

GO Term	**GO ID**	**Associated Genes**	**Group p-value**
Fatty acid biosynthetic process	GO:0006633	*Q8AX86* (↑), *F1QG70* (↓), *Q6PHG4* (↓)	1.5E-5
Biosynthesis of unsaturated fatty acids	KEGG:01040	*Q8AX86* (↑), *F1QG70* (↓)	4.2E-4
Unsaturated fatty acid metabolic process	GO:0033559	*Q8AX86* (↑), *F1QG70* (↓)	4.2E-4
Unsaturated fatty acid biosynthetic process	GO:0006636	*Q8AX86* (↑), *F1QG70* (↓)	4.2E-4