DISSERTATION

ASSESSMENT OF TWO ANTILIPIDEMIC DRUG SUBCLASSES (FIBRATES AND STATINS) ON EMBRYOGENESIS IN TWO MODEL FISH SPECIES (*DANIO RERIO* AND *PIMEPHALES PROMELAS*)

Submitted by

Andrea Kingcade

Department of Environmental and Radiological Health Sciences

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Doctoral Committee:

Advisor: Howard Ramsdell

Marie Legare
Greg Dooley
Deborah Garrity
Dana Winkelman

ABSTRACT

ASSESSMENT OF TWO ANTILIPIDEMIC DRUG SUBCLASSES (FIBRATES AND STATINS) ON EMBRYOGENESIS IN TWO MODEL FISH SPECIES (*DANIO RERIO* AND *PIMEPHALES PROMELAS*)

The antilipidemic drug category is one of many pharmaceutical classes detected in effluent and surface water downstream of wastewater treatment plants. Nine antilipidemic drugs within two subclasses, fibrates (fenofibrate and gemfibrozil) and statins (atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin) are currently prescribed to humans. Embryogenesis in fish is a critical process in development that begins within hours of fertilization and progresses through important stages including gastrulation, neurulation, and organogenesis. To elucidate the effects antilipidemic drugs may have on these sensitive life stages, a mixture exposure study with all nine antilipidemic drugs was performed with zebrafish (*Danio rerio*, ZF) embryos, a developmental biology laboratory model fish species not native to the United States, at three nominal exposure levels: (a) 0.005 µM (Low), (b) 0.05 µM (Medium), and (c) 0.5 µM (High). An additional mixture exposure was performed on fathead minnow (*Pimephales promelas*, FHM) embryos, a toxicological model fish species found in most Colorado streams, at three nominal exposure levels: (a) 0.0005 µM (Ultra Low), (b) 0.005 µM (Low), and (c) 0.05 µM (Medium). Individual drug exposures to ZF embryos were also assessed at two nominal exposure levels: (a) drug-specific environmentally-relevant concentration (ERC Low) and (b) 1 µM (Very High). Test initiation began with blastulating embryos exposed to freshly-prepared exposure solution 4-6 hours post fertilization; ZF studies terminated at 72 hours

and the FHM study terminated at six days post fertilization. Up to 15 observations were divided into four categories and evaluated: developmental toxicity, and muscle, yolk, and cardiovascular abnormalities. Complete mortality was observed in the ZF embryos exposed to 0.05 µM (Medium) and 0.5 µM (High) nominal concentrations in the mixture study and 51% of FHM embryos perished at the nominal 0.05 µM (Medium) exposure level. Developmental delays, delayed dechorionation, abnormal muscle fiber patterns, altered anterior-posterior (AP) axes, and the presence of hemorrhage and pericardial edema significantly increased in FHM embryos exposed to the nominal 0.05 µM (Medium) mixture treatment. Significant decreases in FHM heart rates were observed with the nominal 0.005 µM (Low) exposure compared to unexposed FHM embryos. Developmental delay evaluated as gastrulation defects was recorded in ZF embryos exposed to the nominal 0.5 µM (High) mixture concentration. Abnormal muscle fiber patterns, altered AP axes, abnormal intersegmental vessel development, and the presence of hemorrhage and edema (pericardial and yolk), were significantly increased compared to unexposed ZF embryos in the mixture study. Individual drug exposures did not elicit any toxicity to ZF embryos exposed to gemfibrozil, pravastatin, and rosuvastatin. Six of the nine individual drug exposures (fenofibrate, atorvastatin, fluvastatin, lovastatin, pitavastatin, and simvastatin) exhibited lethal and sublethal effects to ZF embryos. Embryos exposed to lovastatin or simvastatin, the only two prescribed in the prodrug lactone form, exhibited lethal effects in embryos exposed at the nominal 1 µM (Very High) treatment. Twelve sublethal effects were significant in one or more individual drug exposures at the nominal 1 µM exposure level. Abnormal yolk absorption by developing ZF embryos exposed to simvastatin at the nominal 2.4 x 10^{-5} µM (ERC Low) treatment was the only significant effect observed at an environmental concentration. Collectively, these observations illustrate that (a) embryos are sensitive to

antilipidemic drug exposures during embryonic stages of development, (b) differences in species sensitivities occurred, and (c) differences between mixture and individual exposures of drugs were observed. These significant sublethal phenotypes would likely impact individual fish development and potentially the population as well if environmental concentrations increased. This model represents a potential tool for assessing sensitive, sublethal effects of pharmaceuticals in the environment.

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CHAPTER 1

BACKGROUND

Pharmaceuticals and personal care products (PPCPs) are consumed and used regularly in our society. Of the PPCPs that are ingested, they or their metabolites are excreted by the human body into the sewage system (Xia & Bhandari, 2005). Sewage systems transport waste to waste water treatment plants (WWTPs) where such waste is processed before the treated material (effluent) is released into the environment. The Federal Water Pollution Control Act of 1948 was amended in 1972 and 1977 (collectively referred to as the Clean Water Act). These amendments in part govern the regulation of these discharges into navigable waters.

The detection of pharmaceuticals in the terrestrial and aquatic environment has been documented for over 25 years in countries around the world. Studies have shown that PPCPs were not completely removed in waste water treatment plant processing (Drewes, Heberer, Rauch, & Reddersen, 2003; Johnson, Belfroid, & Di Corcia, 2000; Keller, Xia, & Bhandari, 2003). These WWTPs employ a variety of methods for treating sewage including biodegradation and chlorination. However, biodegradation has been considered less effective, or as having no effect, on PPCP removal prior to disposal into the environment (Gros, Petrovic, & Barcelo, 2009; Johnson et al., 2000; Keller et al., 2003). Some WWTPs use biofilm reactors in the biodegradation process, which generally allow biofilm to grow and digest chemical compounds (Nicolella, van Loosdrecht, & Heijnen, 2000). These reactors can be negatively charged so neutral and positively-charged compounds theoretically sorb to the film and negatively-charged compounds sorb more slowly due to electrostatic repulsion of the film and anionic molecules (Carlson & Silverstein, 1998; Riml, Worman, Kunkel, & Radke, 2013).

Another commonly used final disinfection step for treated waste water, chlorination, increased sublethal toxicity of PPCPs in surface water. Exposures of halogenated PPCPs resulted in antiandrogenic effects in laboratory studies (Bulloch et al., 2012).

Therefore, WWTP effluent represents a common source of PPCPs entering the environment (Calamari, Zuccato, Castiglioni, Bagnati, & Fanelli, 2003; Ramirez et al., 2009). Many PPCPs have been found in the aquatic environment because many are polar under environmental conditions, which makes it challenging for WWTPs to effectively treat and remove them prior to effluent release (Conley, Symes, Schorr, & Richards, 2008). Runoff from land with applied biosolids and subsurface transport also help PPCPs move from soil to groundwater (Sangsupan et al., 2006; Topp et al., 2008). Other factors that influence PPCP availability in the environment include geomorphology, hydrology, and water chemistry (Acuña et al., 2015).

A single drug's presence as the sole contaminant in surface water is quite unlikely. Some argue that pharmaceutical risk assessment should also mandate a mixture study be included for this reason (Brain et al., 2004; Daughton, 2003; Emmanuel, Perrodin, Keck, Blanchard, & Vermande, 2005). Because community demographics and the varied WWTP processes generate mixtures of PPCPs that may not be similar, understanding the impacts of PPCP mixtures is important. Also critical is the role each individual drug has in that mixture because that knowledge also assists managers and risk assessors, for example, in prioritizing a drug's removal from toxic effluent.

Over 30 pharmaceutical classes have been detected in effluent discharged into surface waters and at least 11 pharmaceutical classes have been identified as toxic to aquatic organisms (Brausch et al., 2012). The antilipidemic drug class is one of these toxic classes (Suárez,

Carballa, Omil, & Lema, 2008). Within this class exist seven subclasses, including niacins, resins, fibrates, statins, cholesterol absorption inhibitors, and omega-3 fatty acid derivatives. Two subclasses (fibrates and statins) were selected for this project. Statin therapy is popular in human medicine because of the efficacy, tolerance (wide therapeutic index), and profitability (Eichel et al., 2010; Jacobs, Cohen, Ein-Mor, & Stessman, 2013; Langsjoen et al., 2005; Muldoon & Criqui, 1997; Ramsey et al., 2014). Environmentally, the occurrence of one fibrate was widespread and at high concentrations compared to other drug subclasses (Appendix 7); therefore, this subclass was selected. Nine drugs (two fibrates, seven statins) are currently prescribed to humans and were the focus for this project (Table 1.1 and Appendix 1).

Table 1.1

Antilipidemic Drugs Currently Prescribed for Treatment of Human Conditions

Fibrates	**Statins**
Fenofibrate (TriCor)	Atorvastatin (Lipitor)
Gemfibrozil (Lopid)	Fluvastatin (Lescol)
	Lovastatin (Mevacor)
	Pitavastatin (Livalo)
	Pravastatin (Pravachol)
	Rosuvastatin (Crestor)
	Simvastatin (Zocor)

Note. Two subclasses are listed by their generic (brand) names.

Environmental Exposure

Several antilipidemic drugs were identified as of concern to the aquatic environment. Gemfibrozil (GEM) was measured in concentrations higher than 1 µg/L, has a K_{ow} greater than three (Andreozzi, Marotta, & Paxeus, 2003; Fang et al., 2012; Ginebreda et al., 2010; Metcalfe et al., 2003; Sanderson, Johnson, Wilson, Brain, & Solomon, 2003) and is classified as toxic to aquatic organisms (Zurita et al., 2007). Atorvastatin (ATO) and lovastatin (LOV) were determined to be teratogenic based on abnormal gut coiling observed in *Xenopus* (frog) (Fabro,

Shull, & Brown, 1982). Two fibrates were classified as potential endocrine disruptors in fish (bezafibrate, a fibrate no longer prescribed to humans, and GEM) (Mimeault et al., 2005; Velasco-Santamaria, Korsgaard, Madsen, & Bjerregaard, 2011).

All nine antilipidemic drugs selected for this research were detected in the aquatic environment (Appendix 7). Various effluent or surface water concentrations detected in the United States or the nearest country are displayed below (Table 1.2). Concentrations ranged from less than one part per trillion (simvastatin, SIM) up to almost 800 ppt (GEM).

Table 1.2.

Environmental Concentrations Reported in Previous Literature

Sub-class	Drug	Location	Concentration (ng/L)	Reference
Fibrate	Fenofibrate (FENO)	Effluent-Europe	120[a]	(Hernando et al., 2007)(R)
	Gemfibrozil (GEM)	SW-Colorado, SW-USA	73, 790[b]	EPA 2015*, (Kolpin et al., 2002)(R)
Statin	Atorvastatin (ATO)	SW-USA, Effluent-Canada	7.3[b], 77[c]	(Deo 2014) (R) (Lee et al., 2009)
	Fluvastatin (FLUV)	Effluent-Spain	12[d]	(Gros et al., 2012)
	Lovastatin (LOV)	Effluent-Canada	14	(Hernando et al., 2007) (R)
	Pitavastatin (PIT)	Groundwater-India	480	(Jindal et al., 2015)
	Pravastatin (PRAV)	SW-Colorado, Effluent-Canada	73, 59	EPA 2015*, (Hernando et al., 2007) (R)
	Rosuvastatin (ROS)	Effluent-Canada	324[c]	(Lee et al., 2009)
	Simvastatin (SIM)	SW-USA, Effluent-Canada	0.74[b], 1	(Deo 2014) (R) (Hernando et al., 2007) (R)

Note. SW = surface water, (R) = review article, [a]mean concentration, [b]maximum concentration, [c]median concentration, [d]article does not indicate if reported concentration is mean, median, etc., *unpublished data-samples were collected from Colorado and Utah during 2015. Refer to Appendix 7 for a more detailed review.

Mechanism of Action

Fibrates

Within a cell, fatty acids or fibrate drugs (i.e. gemfibrozil) bind to members of the peroxisome proliferator-activated receptor superfamily. Of the three members or subtypes (alpha, beta, and gamma), fatty acids and eicosanoids bind to any of the three; and, fibrates bind to the alpha subtype (PPARα, a transcription factor). This transcription factor-ligand complex heterodimerizes with the retinoid X receptor (RXR). This complex of proteins then binds to peroxisome proliferator response elements (PPREs) found on the DNA, which trigger expression of genes involved with lipid metabolism. Lipoprotein lipase (LPL) transcription will increase to break down triglycerides into fatty acids and glycerol, and very low density lipoproteins (VLDL) are converted to low density lipoproteins (LDL). Thus, fibrates decrease triglyceride concentrations, decrease VLDL, and increase high density lipoproteins (Al-Habsi, Massarsky, & Moon, 2016; Gervois, Torra, Fruchart, & Staels, 2000; Nelson & Cos, 2005; Prindiville, Mennigen, Zamora, Moon, & Weber, 2011; Staels et al., 1998; Varga, Czimmerer, & Nagy, 2011).

The interaction between PPAR alpha and fibrates is contested in fish. Surprisingly, no significant increases in PPAR alpha, beta, or gamma gene expression patterns were observed in juvenile female rainbow trout (*Onchorynchus mykiss*) that were injected with 100 mg GEM/L five times over 15 days (Prindiville et al., 2011). Gene expression of an activator of lipoprotein lipase, apolipoprotein CII, also did not increase in GEM-exposed trout compared to unexposed trout. However, while LPL expression did significantly increase in exposed trout compared to levels measured in unexposed trout; LPL activity in muscle, liver, or adipose tissue (the three areas where measurements were taken) was not significantly affected. Despite

this incongruent gene expression, total lipoprotein levels still decreased (Prindiville et al., 2011). PPAR alpha and gamma gene expression levels were upregulated in zebrafish hepatocytes exposed to 0.5 mM clofibrate and 1 mM clofibrate, respectively, for 24 hours (Ibabe, Herrero, & Cajaraville, 2005). After considering expression of different gene products related to the PPAR pathway, lipid metabolism via any PPAR subtype may be controlled differently between fish and mammals (Feng, Huang, Liu, Zhang, & Liu, 2014; Lindberg & Olivecrona, 1995; Skolness et al., 2012; Velasco-Santamaria et al., 2011), and possibly among fish species as well.

Three members of the triglyceride lipase subfamily (endothelial, hepatic, and lipoprotein lipase) exhibit roles in hyperlipidemia and atherosclerosis (Wang, Li, Sun, Fan, & Liu, 2013). Their molecular structures, expression patterns, and/or activities have been studied in trout (Lindberg & Olivecrona, 1995). Lipoprotein lipase activity in trout was observed but very little or no hepatic lipase-like activity was detected (Lindberg & Olivecrona, 1995). In the developing zebrafish embryo, transcript of lipoprotein lipase was measured between 0.5 hours post fertilization (hpf) and 6 days post fertilization (dpf); hepatic lipase was detected after 3 dpf (Feng et al., 2014).

Statins

In vertebrates, cholesterol is the product of the mevalonate pathway (Santos et al., 2016). The rate-limiting step of this multi-step process is the conversion of hydroxymethylglutaryl (HMG)-Coenzyme A (CoA) to mevalonate by HMG-CoA reductase. Statins are competitive inhibitors of this enzyme. Thus, statins decrease cholesterol synthesis. Also, by reducing LDL and secondarily increasing HDL, decreased triglycerides are observed in humans (Endo, Kuroda, & Tanzawa, 2004; McTaggart & Jones, 2008). In a 54-week human statin exposure, mean

plasma levels of LDL cholesterol and total cholesterol were reduced 42% and 31%, respectively (Andrews, Ballantyne, Hsia, & Kramer, 2001).

Fish Studies

Introduction

Ubiquitous exposure to this drug class in the aquatic environment presents a potential threat to aquatic organisms, including fish. The cholesterol synthesis pathway is highly conserved in metazoan taxa (Santos, 2016) and fish rely on triglycerides for their primary energy storage (Bennett, Weber, & Janz, 2007). Most fish regularly have 2- to 6-fold higher levels of cholesterol compared to mammals (Babin & Vernier, 1989; Larsson & Fange, 1977). and plaque buildup in fish coronary arteries (atherosclerosis) was observed in *Salmo salar* (Atlantic salmon) (Saunders, Farrell, & Knox, 1992). The prevalence and frequency of *S. salar* coronary lesions interestingly correlated with growth rates (not age); therefore, factors responsible for growth may also play a role in cardiovascular health (Saunders et al., 1992).

Transmembrane proteins play essential roles in transporting compounds across cell membranes. Organic anion transporters (similar to mammalian OATP1B1) have been identified in most bilateral organisms, including *Ictalurus punctatus* (catfish) and *Fugu punctatus* (pufferfish) (Hagenbuch & Meier, 2004). Twenty of these genes were also identified in zebrafish (*Danio rerio,* ZF) but the many orthologs compared to humans suggest these proteins may take on additional specific functions in zebrafish (Mihaljevic, Popovic, Zaja, & Smital, 2016). These transporters allow uptake of drugs such as fibrates and statins for metabolism (Popovic, Zaja, Fent, & Smital, 2013). Of the two statin forms (acid or lactone), the nonpolar lactone form may also diffuse across membranes. These two movements of drugs

across membranes contribute to the disposition of statins and other drugs within an animal (Hagenbuch & Meier, 2004; Skottheim, Gedde-Dahl, Hejazifar, Hoel, & Asberg, 2008).

Environmentally-Relevant Studies

Exposure to environmentally-relevant concentrations (ERCs) and higher concentrations of antilipidemic drugs have exhibited effects on fish. GEM was bioconcentrated in goldfish (*Carassius auratus*) blood (bioconcentration factor of 500) after 14 days of exposure at a measured concentration of 0.34 µg GEM/L (Mimeault et al., 2005). GEM was considered a potential endocrine disruptor based on these two goldfish experiments because plasma testosterone levels were reduced >50% (findings of two separate exposure studies: one lasting 96 hours and the other lasting 14 days). Gene expression levels of steroidogenic acute regulatory (StAR), proteins involved with the rate-limiting step of steroidogenesis, were also decreased after just 96 hours but not after 14 days, which suggested the decreased testosterone was not solely due to the delay in cholesterol delivery. Mimeault et al. (2005) suggested the nine-month gap between the two experiments correlated with seasonal differences that may have affected fish sensitivity to environmental contaminants and to StAR expression levels (Mimeault et al., 2005).

Fibrates were also classified as potential endocrine disruptors in fish based on a 21-day bezafibrate dietary exposure to male zebrafish (Velasco-Santamaria et al., 2011). Significant reductions in plasma cholesterol and 11-ketotestosterone were observed in fish exposed up to 200 µg/L bezafibrate. Expression levels of genes associated with steroidogenesis were altered, which led to spermatogenesis defects (Velasco-Santamaria et al., 2011).

Fathead minnows (*Pimephales promelas*) were also exposed to the same environmentally-relevant concentration of gemfibrozil as in the goldfish study (Mimeault et al.,

2005). Cholesterol, triglycerides, and sex steroids were not significantly or consistently affected at this concentration and only a slight decrease in fecundity was observed at a higher tested concentration of 1,500 µg GEM/L after a 21-day exposure (Skolness et al., 2012). FHM may be less sensitive to GEM at the adult stage.

Studies in Fish Exposed to Concentrations Exceeding Those Detected in the Environment

Exposure to concentrations that are not environmentally-relevant have also been evaluated. The median lethal concentrations (LC_{50}) in zebrafish after 72 hours of exposure beginning at 1-4 days post fertilization (dpf) were determined for ATO (469 µg ATO/L) and fenofibrate (FENO) (1.59 mg FENO/L) (Chen et al., 2017). The LC_{50} for GEM in zebrafish after 96 hours of exposure beginning at three hours post fertilization was 11 mg GEM/L (Henriques et al., 2016). Clofibric acid, a fibrate metabolite detected in the environment in µg/L concentrations, significantly altered sperm motility at 1 mg/L and decreased sperm counts at 10 µg/L in adult FHM (Runnalls, Hala, & Sumpter, 2007).

The mevalonate pathway was determined to be critical for proper speed and direction of primordial germ cells (PGCs) migrating through blastulating zebrafish embryos. Primordial germ cells are the precursor cells of gametogenesis for the organism. When zebrafish were exposed to 6 mg ATO/L beginning at 2-4 hpf, 90% of ectopic PGCs were detected at the 3-somite stage (<24 hpf) and >50% were still located ectopically 24 hours later (<50% eventually migrated to the correct location). Less than 10% of PGCs ectopically migrated in the unexposed embryos. A median effective concentration (EC_{50}) of 4 µM ATO was determined. Exogenous addition of mevalonate, farnesol, or geranylgeranyiol rescued this ectopic PGC migration but other compounds in this pathway, such as squalene, did not rescue this ectopic migration. SIM treatment of zebrafish embryos produced a similar pattern of ectopic migration

and rescue but was more severe compared to ATO; no EC_{50} was reported. Therefore, proper PGC migration appeared to depend on this pathway (Thorpe, Doitsidou, Ho, Raz, & Farber, 2004).

Yolk defects and potential endocrine effects have also been explored (Raldúa, André, & Babin, 2008). The upper layer of yolk cells is arranged in a syncytial fashion and is referred to as the yolk syncytial layer. This portion of the yolk may have multiple roles for the developing zebrafish embryo, including transferring nutrients and hormones stored in the yolk during oogenesis and secreting substances such as apolipoproteins. These hormones may impact metabolism and organogenesis in the embryo. Embryonic and larval environments can interfere with the yolk syncytial layer and profound effects of a measurable degree may be observed regardless of whether the zebrafish is exhibiting retardation in growth. Small-sized ZF larvae resulted when exposed to 5 mg GEM/L or another fibrate no longer prescribed to humans, clofibrate (0.75 mg/L clofibrate), as exposure to either drug resulted in insufficient yolk absorption. Mechanical strain was then likely placed on the embryonic axis, which resulted in observed effects such as: delayed swim bladder inflation, delayed digestive system formation, slowed locomotion, and delayed feeding of exogenous food. Vasculogenesis and muscle fiber organization and striation were also disrupted with clofibrate exposure. Heart defects (e.g., pericardial edema, morphology, contractility, atrium position, and chamber length) were recorded at 3-4 dpf at 0.75 mg/L clofibrate (Raldua et al., 2008).

In a preliminary laboratory study, FHM were exposed to 0.5 or 2.2 mg GEM/L (nominal). Growth and length were evaluated at the end of a 28-day exposure in 60-day old FHM (Cadmus and Jefferson 2016). Despite no significant differences noted in any observation (Supplemental S-6), similar to other researchers' conclusions (Raldua et al., 2008), there may

be impacts other than growth retardation. Thus, earlier stages of development were pursued with antilipidemic exposures.

A study investigating a fibrate and a statin, and a separate mixture study involving one statin and one fibrate exposure, were performed in adult ZF. Within seven days of exposure to GEM (380 ng GEM/L), significant genotoxic effects were measured; at 14 days of exposure, DNA repair was evident (Rocco et al., 2012). Similar effects were seen in a statin exposure within five days of exposure and with similar DNA repair time (13 ng ATO/L) (Rocco et al., 2012). In a separate study, adult ZF were exposed to GEM, ATO, or a mixture of both GEM and ATO in their diet for 30 days (16 µg GEM/g fish or 0.53 µg ATO/g fish, equivalent to human dosages of each drug). Levels of cholesterol were measured from whole-body lipid extracts and were found to be significantly lower in the single and mixture exposures (13%-24%). Triglyceride levels were also significantly reduced in the female ZF (30%-37%) when measured from the same lipid extracts in the single and mixture exposures. When exposed only to GEM, males exhibited significant declines in their whole-body triglyceride levels as well. A significant increase in triglyceride content was observed in males exposed to ATO and the mixture of both antilipidemic drugs. It was suggested that females are more sensitive to atorvastatin because triglycerides play a role in oogenesis (Al-Habsi et al., 2016).

Goals of the Project

The primary goal of this project was to comprehensively evaluate and identify lethal and sublethal effects from individual or mixture drug exposures during the embryonic stage of development via a rapid screening approach in two fish species, zebrafish and fathead minnows. ZF are a well-established model organism commonly used in molecular and developmental laboratories. This nonindigenous, tropical fish is ubiquitously studied and

offers many advantages to researchers including transparent embryos, rapid life cycles, large clutches, and easy culturing techniques. Their genome has been fully sequenced and many transgenic and mutant lines are available. ZF are also a model for lipid research (Hölttä-Vuori et al., 2010). FHM are ubiquitously used in aquatic toxicology laboratories as a standard organism for effluent toxicity studies; are naturally found across most of North America including in Colorado; and like ZF, also have transparent embryos and relatively rapid development (Nico, Fuller, & Neilson, 2019; USEPA, 1996).

This project focused on embryogenesis because this life stage is a critical time when cells differentiate into structures and tissues. Also, at this stage, sublethal effects may be evident at very low exposure concentrations (Parrott & Blunt, 2005). ZF embryo research is also gaining popularity as a substitute for adult animal testing because ZF embryos up to 5 dpf do not fall under animal welfare regulations (Piña et al., 2018). Our observations were thus centered around developing structures including those in the cardiovascular and muscular systems. Yolk morphology and successful development past gastrulation were also assessed in both fish species.

Effects of fluvastatin, lovastatin, pitavastatin, pravastatin, and rosuvastatin have not been studied in zebrafish embryos and only one mixture study involving GEM (mixed with other PPCP classes) has been studied in FHM embryos (Parrott & Bennie, 2009). Therefore, this research addresses a gap in the research by: (a) furthering the knowledge on how all these drugs toxicologically compare to each other; (b) examines how individual drugs impact embryogenesis; (c) compares adverse effects found in the current research with those found in previous studies; and, (d) explores the utility of using sublethal effects in fish embryos as a sensitive model for the assessment of wastewater-associated pharmaceutical compounds. If

fish are exposed to concentrations of pharmaceuticals that prevent the animals from properly developing into sexually-mature adults, then protective measures should be considered to protect aquatic life.

CHAPTER 2
MATERIALS AND METHODS

These studies focused on identifying relevant effects on fish embryonic development when exposed to antilipidemic drugs (Appendix 9). Two species of fish, *Danio rerio* (zebrafish, ZF) and *Pimephales promelas* (fathead minnow, FHM), were exposed to antilipidemic drugs for 72 hours or six days, respectively, during embryogenesis. All fish procedures were conducted in accordance with protocols approved by the Colorado State University Institutional Animal Care and Use Committee (IACUC #17-7606A).

Source of Animals

Adult ZF were raised and maintained in a laboratory in the Department of Biology at Colorado State University (CSU). Fish were fed two to three times daily with either live, freshly-hatched brine shrimp, GEMMA Micro 300 (GM-300, Skretting, Westbrook, ME), or TetraMin flake food. They were kept segregated by sex, in flow-through, recirculating conditions. Automatic pumps supplied solutions of Sea Salt (Instant Ocean, Blacksburg, VA) and sodium bicarbonate (Fisher Scientific, Hampton, NH) to adjust reverse osmosis water to a target pH of 7.2 and conductivity of 550 µS (ZF system water). Reverse osmosis water originated from City of Fort Collins chlorinated tap water. Automatic heaters maintained a system target temperature of 28 °C.

Adult transgenic zebrafish [Tg(*fli-1*:GFP);(*gata-1*:RFP)] had two transgenes inserted into the genome linked to tissue-specific gene promoter constructs: green fluorescent protein (GFP) and red fluorescent protein (RFP) (Lawson & Weinstein, 2002; Traver et al. 2003). Heterozygous parents were mated and due to the heterozygosity, only a proportion of embryos expressed the transgenes. The GFP transgene was expressed in endothelial cells (utilizing the *fli*

promoter specifically expressed in this cell type) as early as 24 hpf and the RFP transgene was expressed in red blood cells (utilizing the *gata* promoter specifically expressed in this cell type) as early as 48 hours post-fertilization (hpf).

Adult FHM were purchased from Aquatic Biosystems (Fort Collins, CO) and maintained in the aquatic toxicology laboratory of Colorado Parks and Wildlife (Fort Collins, CO). Fish were fed ground trout chow enriched with spirulina three times daily. They were kept segregated by sex, in flow-through conditions with aerated, moderately-hard water (FHM system water) (ASTM, 1996). The FHM system water was prepared by mixing dechlorinated tap water, supplied by the City of Fort Collins, and water from a well located on the property of Colorado Parks and Wildlife. Automatic heaters maintained a system target temperature of 25 °C. Substrate (longitudinally-cut sections of three-inch polyvinyl chloride pipe) was placed in each culture tank to provide hiding spots so as to more closely mimic breeding conditions, and to decrease stress. Black waterproof corrugated polypropylene sheets (correx) or pink Styrofoam was placed between the tanks to reduce stress.

Source of Drugs

Of the nine drugs, FENO, GEM, ATO, FLUV, and LOV were purchased from TCI America (Portland, OR), PRAV was purchased from Toronto Research Chemicals (Ontario, Canada), and the remaining three (PIT, ROS, and SIM) were purchased from BOC Sciences Inc. (Shirley, NY). All were purchased in their solid form of at least 98% purity in Spring 2018 and stored according to the manufacturer's recommendations.

Breeding and Embryo Collection

Up to three females and up to four males of either species were placed together in separate breeding-specific tanks the evening prior to embryo collection. The ZF were placed in a

plastic mesh container within their breeding tanks that the eggs could pass through but that the adult fish could not. ZF were held overnight in static system water. Substrate was placed into each of the FHM breeding tanks to allow egg deposition; black correx was placed between the FHM breeding tanks. FHM were held overnight in flow-through system water.

Embryos were collected the following morning (day of test initiation) from healthy clutches (at least 20 embryos per breeding tank, no more than five unfertilized embryos) and rinsed with their respective system water. In any study, at least three breeding tanks must have had clutches to ensure sufficient genetic diversity. If not, fish were returned to their culture tanks and breeding was attempted again three to five days later. FHM embryos were removed from the substrate by gently rubbing them off with moist fingers into system water. Both FHM and ZF embryos were gently rinsed with system water in strainers. ZF embryos were then transferred to 100-mm, untreated, polystyrene petri dishes (Fisher Scientific, Catalog #FB0875712, Hampton, NH) with E3 media (ZF dilution water). The FHM embryos were transferred via a double-insulated carrier to the CSU laboratory in 600-ml tissue culture flasks with moderately hard reconstituted water (ASTM, 1996) (FHM dilution water) then placed in plastic petri dishes with FHM dilution water.

Experimental Design

Dilution Waters

Dilution waters used for the studies were prepared by mixing salts with reverse osmosis water (ZF) or Type 1 water (FHM, ASTM, 2018). Both dilution waters used potassium chloride (ACS grade) (Fisher Scientific, Hampton, NH), sodium bicarbonate (ACS grade) (Fisher Scientific, Hampton, NH or J.T. Baker Chemical, Radnor, PA), and magnesium sulfate (EM Science, Gardena, CA or Sigma-Aldrich, St. Louis, MO). The ZF dilution water (E3 medium)

additionally used sodium chloride (Biological grade) (Fisher Scientific, Hampton, NH) and calcium chloride (ACS grade) (Fisher Scientific, Hampton, NH) and was pH-adjusted to 7.3 ± 0.2 with hydrogen chloride. The FHM dilution water additionally used calcium sulfate (Mallinckrodt Analytical Reagent, Staines-Upon-Thames, UK) and was aerated. The ZF dilution water was made according to the standard operating procedure of the CSU laboratory with the final nominal concentrations of 4.9 mmol/L NaCl, 0.17 mmol/L KCl, 0.32 mmol/L $CaCl_2$, 1.1 $NaHCO_3$, and 0.33 mmol/L$MgSO_4$. The FHM dilution water (moderately hard reconstituted water) had a hardness of 95 mg/L $CaCO_3$ ± 10% and an alkalinity of 60 mg/L $CaCO_3$ ± 10% (Standard Method 1997a, Standard Method 1997b).

Embryo Sorting

Collected embryos were sorted and unfertilized, unhealthy (asymmetrical cleavage, abnormal morphology), or older (greater than 4-6 hpf, gastrula or later stage) embryos were discarded. Gentle attempts to separate FHM embryos that adhered together was performed with dissecting probes but if the embryos were older, their adherence was too strong and they were discarded. Only individual FHM embryos were evaluated. Five eligible embryos at a time were then transferred to unlabeled, polystyrene plastic, 60-mm petri dishes (Fisher Scientific, Catalog #FB0875713A, Hampton, NH) filled with appropriate dilution water. This was repeated three more times until 20 embryos were in each plastic petri dish. The developmental stage was verified again prior to exposure to the drugs. Embryos undergoing gastrulation were replaced.

Solution Preparation and Embryo Exposure

Up to four exposure levels were evaluated. The "ERC Low" concentrations were based upon surface water (GEM) or effluent (remaining eight drugs) concentrations reported in the literature, and all other tested concentrations were standardized using molar concentrations for

each drug at each exposure level (Tables 2.1 and 2.2). Effluent concentrations recorded nearest to the state of Colorado were selected as the basis for eight of the nine ERC Low drug concentrations. Due to the wide range of gemfibrozil effluent concentrations recorded in the environment (Appendix 7), the maximum U.S.-recorded surface water concentration was selected as the basis for the ERC Low GEM concentration.

Drug(s) were combined into a single volumetric flask for each study and only solubilized with dimethyl sulfoxide (DMSO) on the morning of each test initiation to create a stock. To have DMSO stocks for lower concentrations, this stock was immediately diluted further with DMSO. All stocks were stored at 4 °C in the dark when not in use. Each stock was added dropwise into a vortex of dilution water and mixed for 15 minutes on a magnetic stir plate at room temperature. Embryos from each unlabeled plastic petri dish were randomly assigned and transferred to a testing chamber (60 x 15 mm borosilicate glass petri dishes, VWR #75845-542, Radnor, PA) with 10 ml of exposure solution using a P1000 tip that had been cut wider to avoid embryo shearing. Once all embryos were transferred, testing chambers with embryos were incubated (ZF: 28 °C; FHM: 25 °C) in the dark for the study duration (ZF: 72 hpf; FHM: 6 dpf) except during data collection or test renewal. On Day 0 of the FHM mixture study, embryos remained at room temperature (23 °C) for approximately two hours after test initiation while the incubator adjusted to the target temperature. Once resolved, embryos were incubated at 25 °C ± 1 °C for the duration of the study.

Table 2.1

Target Concentrations Tested in Mixture Studies

Drug	Target Concentrations			
	Ultra Low[a] µM	Low[a, b] µM	Medium[a, b] µM	High[b] µM
Fenofibrate (FENO)	0.0005 (0.18 µg/L)	0.005 (1.8 µg/L)	0.05 (18 µg/L)	0.5 (181 µg/L)
Gemfibrozil (GEM)	0.0005 (0.12 µg/L)	0.005 (1.2 µg/L)	0.05 (12 µg/L)	0.5 (125 µg/L)
Atorvastatin (ATO)	0.0005 (0.28 µg/L)	0.005 (2.8 µg/L)	0.05 (28 µg/L)	0.5 (280 µg/L)
Fluvastatin (FLUV)	0.0005 (0.21 µg/L)	0.005 (2.1 µg/L)	0.05 (21 µg/L)	0.5 (206 µg/L)
Lovastatin (LOV)	0.0005 (0.20 µg/L)	0.005 (2.0 µg/L)	0.05 (20 µg/L)	0.5 (202 µg/L)
Pitavastatin (PIT)	0.0005 (0.21 µg/L)	0.005 (2.1 µg/L)	0.05 (21 µg/L)	0.5 (211 µg/L)
Pravastatin (PRAV)	0.0005 (0.21 µg/L)	0.005 (2.1 µg/L)	0.05 (21 µg/L)	0.5 (213 µg/L)
Rosuvastatin (ROS)	0.0005 (0.24 µg/L)	0.005 (2.4 µg/L)	0.05 (24 µg/L)	0.5 (241 µg/L)
Simvastatin (SIM)	0.0005 (0.21 µg/L)	0.005 (2.1 µg/L)	0.05 (21 µg/L)	0.5 (210 µg/L)

Note. Each target exposure concentration is shown in two units (µM and µg/L). The concentration of DMSO in each exposure was standardized (0.01%). [a] Target concentrations from the FHM mixture study, [b] Target concentrations from the ZF mixture study, ZF = zebrafish, FHM = fathead minnow.

Table 2.2

Target Concentrations Tested in Individual Studies

Drug	ERC[a] µM	Target Concentrations	
		ERC Low[b] µM	Very High µM
Fenofibrate (FENO)	2.8×10^{-4} (0.1 µg/L)	2.8×10^{-3} (1 µg/L)	1 (361 µg/L)
Gemfibrozil (GEM)	3.2×10^{-3} (0.8 µg/L)	3.2×10^{-2} (8 µg/L)	1 (250 µg/L)
Atorvastatin (ATO)	1.4×10^{-4} (0.08 µg/L)	1.4×10^{-3} (0.8 µg/L)	1 (559 µg/L)
Fluvastatin (FLUV)	2.4×10^{-5} (0.01 µg/L)	2.4×10^{-4} (0.1 µg/L)	1 (411 µg/L)
Lovastatin (LOV)	2.5×10^{-5} (0.01 µg/L)	2.5×10^{-4} (0.1 µg/L)	1 (404 µg/L)
Pitavastatin (PIT)	2.4×10^{-5} (0.01 µg/L)	2.4×10^{-4} (0.1 µg/L)	1 (421 µg/L)
Pravastatin (PRAV)	1.4×10^{-4} (0.06 µg/L)	1.4×10^{-3} (0.6 µg/L)	1 (425 µg/L)
Rosuvastatin (ROS)	6.2×10^{-4} (0.3 µg/L)	6.2×10^{-3} (3 µg/L)	1 (482 µg/L)
Simvastatin (SIM)	2.4×10^{-6} (0.001 µg/L)	2.4×10^{-5} (0.01 µg/L)	1 (419 µg/L)

Note. Each target exposure concentration is shown in two units (µM and µg/L). The concentration of DMSO in each exposure was standardized (0.01%). [a] ERCs (effluent) from Table 1.2 were rounded to the nearest whole number except surface water concentration for GEM was used. No effluent concentrations were measured for PIT so the lowest but most frequent statin concentration (10 µg/L) was used. [b] ERC Low was targeted to be 10 times the ERC, ERC = environmentally-relevant concentration.

Remaining Study Details

Each exposure replicate was renewed approximately every 24 hours with freshly prepared exposure solutions using the same DMSO stocks from test initiation and Pasteur pipets. Each concentration group was evaluated with four replicates and the DMSO concentration was standardized in all groups to 0.01% (including the solvent control, referred to as "Control" from here on). Testing chambers, flasks, and cylinders were washed with detergent, nitric acid, and

acetone, and then heavily rinsed with ASTM Type 1 water (ASTM, 2018) between studies. Testing chambers, plastic micropipet tips, and analytical vials were prerinsed three times with solution (DMSO, exposure solution, etc.) prior to use. Pipets and glassware, including flasks and cylinders, were pre-rinsed and post-rinsed with Type 1 water daily. Water quality chemistry values including temperature (digital and alcohol thermometers), pH (Corning 340), and dissolved oxygen (EcoSense ODO200) were recorded daily in aliquots of freshly prepared solutions and old exposure solutions (Appendix 4) in all studies except for FENO (pH only). Temperatures were recorded in one replicate per treatment prior to removal of the testing chambers from the incubator. Calibration of probes and micropipets occurred daily.

Observation of Developmental Abnormalities

Fifteen observations in the ZF studies and ten observations in the FHM mixture study encompassed four categories: developmental toxicity and muscular, yolk, and cardiovascular abnormalities. These observations were generally made before daily renewal of test solutions. All observations evaluated at test termination were made under single-blind conditions by transferring embryos from their labeled testing chambers to unlabeled 3x3 glass depression slides (total of nine depression wells).

Developmental Toxicity: Mortality, Developmental Progress, Dechorionation

Mortality was assessed daily in every study. Before the heart developed, mortality was recorded when most cells were opaque, dark, and the membranes were degrading. After cardiac contractions began, mortality was also recorded when there was no contraction during a 20-second observation period. Cumulative mortality was analyzed.

Developmental progress was evaluated based on ZF embryos completing gastrulation successfully and FHM exhibiting signs of progressing development. Successful completion of

gastrulation, a critical embryonic stage when three germ layers are ultimately formed and body axes are established, was measured at approximately 24 hpf in all ZF studies. Embryos were stored temporarily in a small incubator within the room where this assessment took place to minimize prolonged exposure at room temperature. Gastrulation was scored as complete if the anterior-posterior (AP) axis, somites, and yolk extension were present with a moderate amount of yolk present that had a spherical shape. Unsuccessful completion was evaluated when large amounts of yolk remained without a properly-formed yolk extension or if posterior structures or somites were absent. The most common abnormal appearance of the yolk shape was one that had a kidney bean shape. The proportion of embryos that were evaluated as unsuccessful, in addition to the first 24-hour mortalities, were analyzed. After this was assessed, ZF embryos in each replicate were sorted into two separate dishes of freshly-prepared exposure solution based on their GFP expression (transgenic, T, GFP-expressing or nontransgenic, NT, non-GFP-expressing) using a fluorescence microscope (Olympus SZX12, Center Valley, PA).

Developmental progress was determined by the presence of developing organs or structures on Day 2. Morphologically scoring for gastrulation on Day 1 is challenging as it is difficult to distinguish if embryos are developmentally delayed or simply morphologically a few hours younger than others. Therefore, scoring development on Day 2 avoided this confusion by using more easily-recognized structures. By 48 hpf, FHM embryos should have a complete AP axis, optic cups, somites visibly extending along the axis, and the future heart beginning to form; embryos were evaluated as developmentally progressing or normal. If any of these features were missing, the FHM embryo was evaluated as developmentally delayed. This affected proportion in addition to the first 48-hour mortalities were analyzed.

The number of embryos that dechorionated was recorded on Day 2 of the ZF studies and daily in the FHM study. Any ZF embryos (NT and T) not already dechorionated were manually dechorionated using dissecting probes to gently remove the chorion from around each embryo. The sum of early dechorionation from both NT and T embryos prior to the forced removal was analyzed. Manual dechorionation was necessary in ZF studies to assess muscle abnormalities but was performed with less success in the FHM mixture study. Chorion hardness and the large size of the embryo with minimal perivitelline space resulted in unavoidable damage to embryos. The total number of dechorionated FHM at test termination was analyzed.

Muscle Abnormalities: Touch Stimulus, Birefringence

Muscle fibers formed by 48 hpf in ZF embryos. To assess the function of this organ system in ZF embryos, dechorionated, nontransgene-expressing embryos were placed in a separate "touch chamber," a testing chamber prefilled with freshly-prepared exposure solution. This touch chamber sat on top of two plastic petri dishes. These plastic petri dishes sat on top of an ultra-thin LED light pad dimmed to medium brightness equipped with a calibration scale. Above this set-up, a high-speed camera (Casio Exilim EX-F1 Digital Camera, Tokyo, Japan) was positioned.

Attempts to stimulate a ZF embryo with sound (subsonic to ultrasonic amplified through speakers) or with vibration (via a custom-built circuit using a piezoelectric beeper and a trigger button) were both unsuccessful in 48 hpf and 72 hpf embryos during method development. A touch stimulus was successfully optimized and executed with a dissecting probe gently attempting to touch the dorsoposterior portion of the ZF embryo. Responses were captured at 600 frames per second and maximum velocity (cm/s) was quantified using Tracker software (version 4.11.0, Open Source Physics). When an embryo spontaneously moved in the touch

chamber prior to the manual touch stimulus, it was given a 60-second rest period prior to performing the assay. A minimum of four NT embryos per replicate were each assessed and then transferred to a "holding chamber," which was filled with fresh exposure solution to avoid duplicate measurements. Once all touch assays were complete for a replicate, embryos were moved from the holding chamber back to their testing chambers, which had been filled with fresh exposure solution. If at least four NT embryos were not available, random T embryos were transferred to the NT-testing chamber prior to the start of touch assays to meet the minimum required and those embryos remained with the NT embryos for the remainder of the study. Because manual dechorionation was challenging with FHM embryos and because of the continuous rapid movements of those that had dechorionated, the touch assay was not performed with FHM embryos.

Muscle fiber development was also observed under the microscope with a rotatable polarized lens (birefringence) at test termination. In both dechorionated transgenic ZF and dechorionated FHM embryos, the normal muscle fiber arrangement showed tightly-packed, parallel fibers with bright light passing through. Abnormal patterns that showed dark, disrupted, non-linear, or loosely-packed fibers were recorded.

Yolk Abnormalities: AP Axis, Yolk Area

Yolk absorption was observed via the angle of the AP axis and the amount of yolk remaining, both viewed only in laterally-positioned, dechorionated embryos. Both yolk abnormalities were evaluated at test termination in transgene-expressing ZF and FHM embryos. Any noticeable deviation from the angle between the anterior and posterior ends (180° normally) was evaluated as having an abnormal AP axis. Yolks (including any yolk extension) of up to three dechorionated embryos per replicate selected randomly, if available, were quantified. A

random number for each row and column of the depression slide and fish position within each depression well was generated three times using Excel. Each embryo identified in that manner was then positioned and images were captured with a Retiga QImaging R1 camera and QImaging Ocular software (version 2.0.1.496, Redwood City, CA). Yolk area was quantified with ImageJ (version 1.51j8, National Institute of Health).

Cardiovascular Abnormalities: Vessel Development, Edema, Hemorrhage, Heart Rate

All cardiovascular abnormalities were evaluated in FHM and ZF embryos at test termination, except subintestinal and intersegmental vessels (SIVs and ISVs, respectively), which were only evaluated in T-zebrafish embryos. All embryos were evaluated in the lateral and upright positions. Abnormal SIV development was divided into three categories based on the vascular plexus (or basket) patterning: SIV-underdeveloped (SIV-under), SIV-overdeveloped (SIV-over), and SIV-missing. SIV-under patterns were evaluated when SIV baskets were present but not bilaterally symmetrical (on left-to-right axis), when less than five lateral compartments were present in each basket, or when the basket extended down less than 1/3 of the side of the yolk. SIV-over abnormalities were evaluated when more than three outgrowths extending beyond the ventral-most portion of the basket were present, when they traversed over 3/4 of the side of the yolk (dorsoventral), or there were two rows of compartments (one more dorsal, one more ventral to that on one side of the yolk). SIV-missing was evaluated when SIVs were not present on an embryo. The sum of each proportion of each of these three categories in each replicate resulted in the SIV-total category that was only analyzed statistically, not visually. ISVs were evaluated as abnormal if they were underdeveloped (incomplete growth between the dorsal aorta and the dorsal longitudinal anastomotic vessels) or missing.

The presence of pericardial and yolk edema and hemorrhage were evaluated in both FHM and ZF embryos. When the distance between the heart chambers and the pericardium was obviously increased and anterioventral bowing was present, this was recorded as pericardial edema in dechorionated and chorionated FHM embryos and in dechorionated transgene-expressing ZF embryos. Presence of yolk edema was evaluated by inspecting the space between the yolk and embryo and the ventral side of the yolk. If any swelling was present, a yolk edema was recorded. This was only assessed in the transgene-expressing ZF embryos and dechorionated FHM. The presence of hemorrhage was evaluated in transgene-expressing ZF embryos and both dechorionated and chorionated FHM embryos. Hemorrhage was defined as visibly-pooled blood (under bright field) that was not circulating. The entire embryo was evaluated for the presence of hemorrhage.

Heart rates were quantified in five FHM embryos (dechorionated or chorionated) per replicate at test termination while in the depression wells prior to other observations. Embryos were randomly selected and blindly assessed. The number of beats counted in six second increments was recorded three times per embryo, and each was multiplied by 10 to determine average beats per minute. After heart rates were measured, FHM embryos were anesthetized in their depression wells with 20-50 µL of tricaine methanesulfonate (TMS or MS-222, 2 mg/ml stock) to facilitate remaining observations at test termination.

Image Manipulation

Post-processing of captured images utilized Microsoft Word or PowerPoint (version 1812, Microsoft Office 365 ProPlus). Brightness, color contrast, color saturation, color tone, or sharpness were adjusted to facilitate observations. These adjustments did not alter or affect any analyses.

Test Termination and Pathology

All embryos were terminated after sublethal criteria were assessed utilizing rapid exposure to 6-10% bleach or with a lethal dose of MS-222. Embryos were terminated with MS-222 only when they were to be analyzed pathologically (ZF mixture and FHM mixture studies, only). After termination, embryos collected for histopathological evaluation were submerged in Bouin's solution and transported to Colorado State University's Veterinary Diagnostic Laboratory (Fort Collins, CO). Embryos were analyzed under single-blind conditions.

Fixed embryos were embedded for sagittal sections, processed routinely, serially sectioned at 4 mm (typically 10-20 per embryo), and stained with hematoxylin and eosin for histology. Briefly, mild pericardial effusion was recorded when there was expansion of the pericardial space resulting in anterioventral bowing of the embryo profile. Severe pericardial effusion was subjectively defined as when the mid-sagittal cross-sectional area of the pericardial space was equal or greater than twice the cross-sectional area of the cardiac profile, resulting in severe anterioventral bowing. Skeletal muscle toxicity was recorded when myocyte(s) had many eosinophils present with loss of cross striation and condensing or degrading nuclei. Additional scoring for coelomic effusion and cranial edema was performed but no results are reported here; the analyses are pending.

Statistics

Most results were analyzed as proportions of each abnormality present in the replicate and number alive; data was transformed with the arc sine square root transformation prior to statistical analysis. Touch assays (maximum velocity), yolk areas, and average FHM heart rates were analyzed with each embryo's quantified response. All transformations, analyses, and graphs were performed in GraphPad Prism (version 8.0.1, GraphPad Software). Graphs were box-

whisker plots (box: 25th-75th quartiles with median (single bar) and mean ("+") displayed; whiskers: minimum to maximum values). To indicate significant differences, box brackets with their ends above each of the two groups with the corresponding p value symbols ($p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***), were displayed. Median lethal concentrations (LC_{50}) and half maximal effective concentrations (EC_{50}) were calculated in Microsoft Excel (version 1812, Microsoft Office 365 ProPlus) using log transformed molar concentrations.

Normality (Shapiro-Wilks) was assessed in all studies. When more than two treatment groups were present with normal distribution, heteroscedasticity (Bartlett's or Brown-Forsythe) was also evaluated. If values were normally-distributed with equal variance, the data was considered parametric. If the values were not normally distributed and/or not equal variance, the data was considered nonparametric. If parametric, one-way ANOVA with Tukey's multiple comparison test (single pooled variances) was calculated. If non-parametric, Kruskal-Wallis ANOVA with corrected Dunn's multiple comparison tests were performed. When two treatment groups were present with a normal distribution of values, a parametric t-test (unpaired, $\alpha = 0.05$, two-tailed, assuming equal standard deviations) was performed. If values were not normally distributed, non-parametric Mann-Whitney t-test (unpaired, $\alpha = 0.05$, two-tailed) was performed. Both t-tests used exact calculated p values to determine significant differences.

Analytical Chemistry

Aliquots of exposure solutions were collected once per study. Four to 10 milliliters of freshly-prepared solution and day-old solution from one replicate per treatment group was transferred to prerinsed glass vials with Teflon caps. All samples were stored in the dark at 4 °C.

Detection of all nine drugs in a single run was successfully optimized at Colorado State University's Center for Environmental Medicine Analytical Toxicology Laboratory (Fort

Collins, CO) with Ultra Performance Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (UPLC-MS/MS). A C-18, 2.7 µm particle size, 2.1x100 mm column (Agilent) on an Agilent 1290 LC system with electrospray ionization (ESI) in both positive and negative modes on an Agilent 6460 triple quadropole mass spectrometry was used. During the nine-minute run, initial starting column conditions were 80:20 (A:B) (0.1% acetic acid in water:0.1% acetic acid in acetonitrile) with ending conditions of 20:80 (A:B) at a flow of 0.4 ml/min. The percent change was 7.5% for the first two minutes, 10% for the subsequent 1.5 minutes, and 8.9% for the remaining 4.5 minutes. Agilent MassHunter software (version B.06.00) acquired and analyzed the data. Ions, retention times, conditions of analysis, and chromatograms from one freshly-prepared mixture sample of all nine drugs (using DMSO stock less than five days old) are shown in the Supplemental information (Appendix 5). Due to limited resources, no further method development or quantification took place.

CHAPTER 3

MIXTURE STUDIES

Mixture studies were conducted with two fish species (zebrafish or fathead minnows), with each species exposed to a nine-drug mixture of antilipidemic drugs. Zebrafish (*Danio rerio*, ZF) were exposed at three nominal exposure levels: (a) 0.005 µM (Low), (b) 0.05 µM (Medium), and (c) 0.5 µM (High) for 72 hours beginning with blastulating embryos four hours post fertilization (hpf) or younger. Fathead minnows (*Pimephales promelas*, FHM) were exposed at three nominal exposure levels: (a) 0.0005 µM (Ultra Low), (b) 0.005 µM (Low), and (c) 0.05 µM (Medium) for six days beginning with blastulating embryos six hours post fertilization or younger. The ZF mixture study was performed first and due to high embryo mortality observed in the groups treated at the highest exposure concentrations, lower concentrations were selected for the FHM mixture study. Fifteen observations (sublethal and lethal) were evaluated in the ZF study and ten were assessed in the FHM study. Because FHM were not transgenic, vessel development was not assessed.

Water chemistry values for both mixture studies ranged from pH 7.3 up to 8.2, dissolved oxygen never fell below 8 mg/L, and testing temperatures were within 1 °C of the target temperature (28 °C-ZF, 25 °C-FHM) once incubation began (Appendix 4). On Day 0 of the FHM mixture study, all embryos remained at room temperature (23 °C) for approximately two hours longer after test initiation while the incubator adjusted to the target testing temperature.

Developmental Toxicity

The first category of observations assessed was developmental toxicity, which included cumulative mortality, developmental progress, and dechorionation. This category was assessed

in both transgene- and nontransgene-expressing embryos. Mortality was assessed in both ZF and FHM mixture studies daily and cumulative mortality was calculated. Embryos were considered alive when animal cells appeared non-opaque with defined cell membranes and, if the heart had formed, a visible heartbeat. Developmental progress was determined by successful completion of gastrulation in zebrafish on Day 1 or evidence of organogenesis in fathead minnows on Day 2. Presence of a fully-developed anterior to posterior body axis, moderate volume and round shape to yolk with a yolk extension, somitogenesis, and beginnings of future heart and eye development were required for an embryo to be evaluated as developmentally-progressing. If one or more of these features were absent, an embryo was evaluated as exhibiting gastrulation defects (in zebrafish) or developmentally delayed (in fathead minnows). Finally, dechorionation was determined by recording how many zebrafish embryos naturally dechorionated early before forced dechorionation took place on Day 2 or how many fathead minnow embryos naturally dechorionated by Day 6. This hatching process naturally occurs once an embryo develops a hatching gland and that gland releases choriolytic enzymes which degrade the chorion (Korwin-Koassakowski 2012). Thus, embryos that arrest or are slow to develop are often unable to dechorionate at the appropriate time (ZF: 2 dpf; FHM: 5 dpf). Therefore, the percentage of naturally dechorionated embryos is an additional measure of developmental progress.

Complete lethality occurred in the nominal 0.05 µM (Medium) and in the nominal 0.5 µM (High) exposure groups of the ZF mixture study (Figure 3-1). Only 2.5% mortality occurred in the nominal 0.005 µM (Low) exposure group. Mortality was significantly different between the Control group and the Medium and High exposure groups. Greater than 50% of the zebrafish embryos exhibited gastrulation defects during the mixture study (Figure 3-2). Gastrulation was significantly disrupted in 87% of ZF embryos exposed to the nominal 0.5 µM (High) exposure

group compared to the 1% of affected embryos in the nominal 0.005 µM (Low) group and 1% of those in the Control group. These gastrulation defect occurrences were associated with those observed in ZF mortality (four replicates) (Figure 3-3). The incidence of early dechorionation in ZF embryos was not significantly affected when exposed to a mixture of nine antilipidemic drugs (Appendix 2).

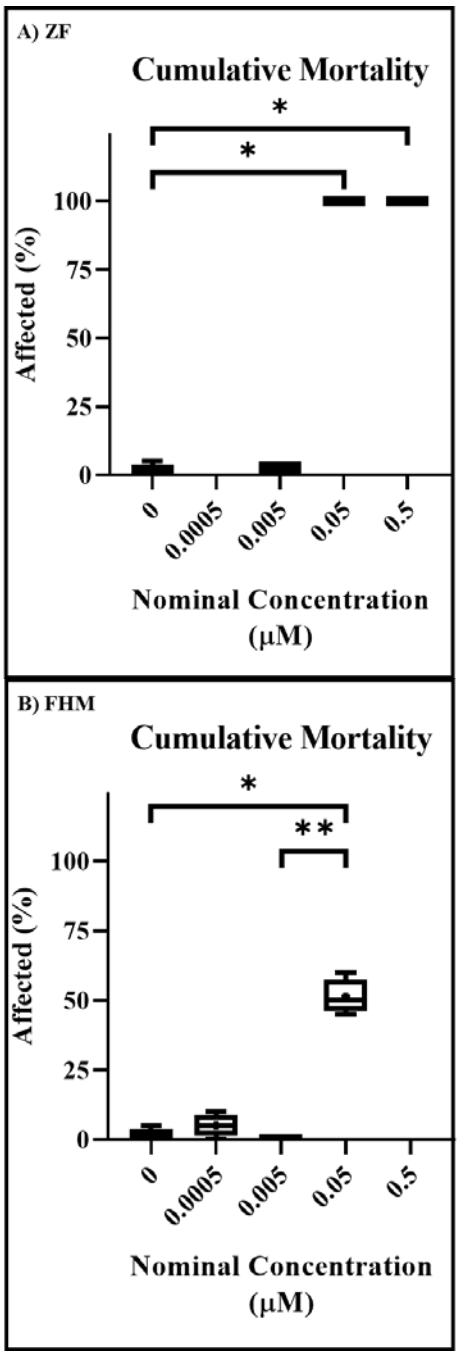

Figure 3-1. Cumulative Mortality in Mixture Studies. Percent of embryos that died (cumulative mortality) from (A) ZF and (B) FHM mixture exposure studies is displayed. Transgene- and nontransgene- expressing zebrafish embryos were evaluated. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25^{th} - 75^{th} percentiles), and whiskers are minimum and maximum values. Refer to Appendix 2 for corresponding morphology pictures. $p \leq 0.05$ (*); $p \leq 0.01$ (**)

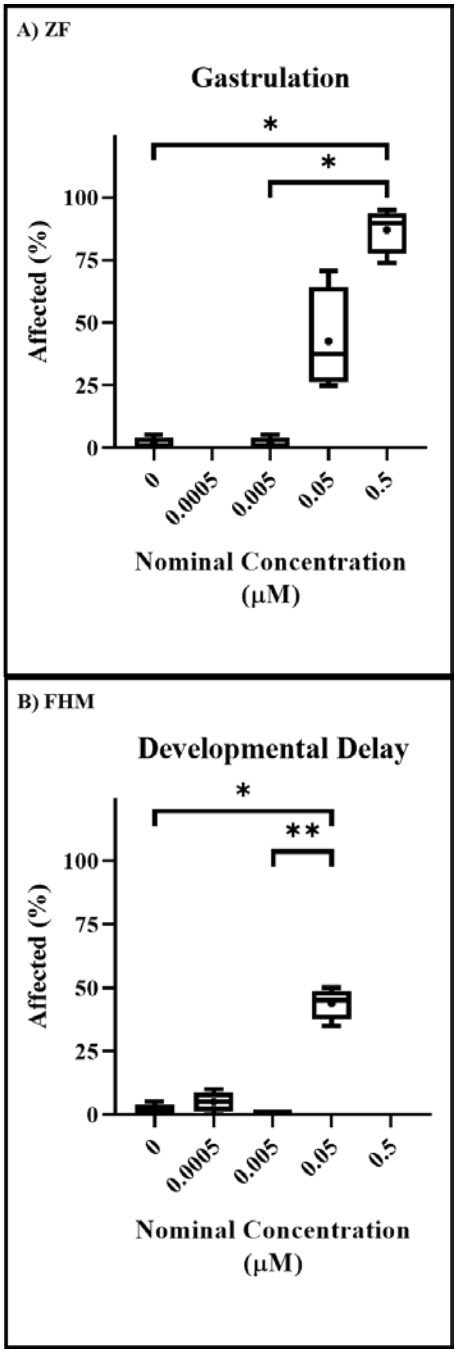

Figure 3-2. Developmental Progress in Mixture Studies. Percent of embryos with developmental delay from (A) ZF and (B) FHM mixture exposure studies is displayed. Progress was evaluated based on presence of gastrulation defects in transgene- and nontransgene-expressing ZF embryos or evidence of delayed organogenesis in FHM embryos. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25^{th} - 75^{th} percentiles), and whiskers are minimum and maximum values. Refer to Appendix 2 for corresponding morphology pictures. $p \leq 0.05$ (*); $p \leq 0.01$ (**)

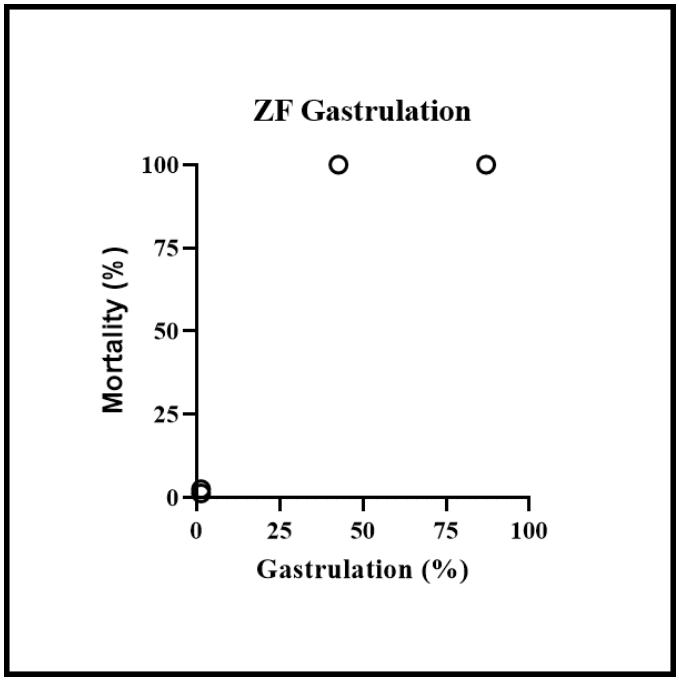

Figure 3-3. Association in the ZF Mixture Study: Developmental Toxicity. Association with the incidences of developmental delay (gastrulation defects) and cumulative mortality from the ZF mixture study is displayed with values from four replicates.

Partial lethality (51%) occurred in the nominal 0.05 µM (Medium) exposure group of the FHM mixture study. No mortality occurred in the nominal 0.005 µM (Low) exposure group and 1% occurred in the Control group. Mortality was significantly different between the Control group and the nominal 0.05 µM (Medium) and the nominal 0.005 µM (Low) exposure groups. Forty-four percent of FHM embryos experienced developmental delay in the nominal 0.05 µM (Medium) exposure group (Figure 3-2). This was significantly different from fathead minnow embryos that experienced delay in the nominal 0.005 µM (Low) (0%) and Control (1%) exposure groups. These developmental delay occurrences were associated with those of FHM dechorionation and 100% with those observed in mortality (four replicates) (Figure 3-4). A significant number of FHM embryos did not dechorionate on their own by test termination in the

nominal 0.05 µM (Medium) exposure group compared to those in the nominal 0.0005 µM (Ultra Low) exposure group (Figure 3-5).

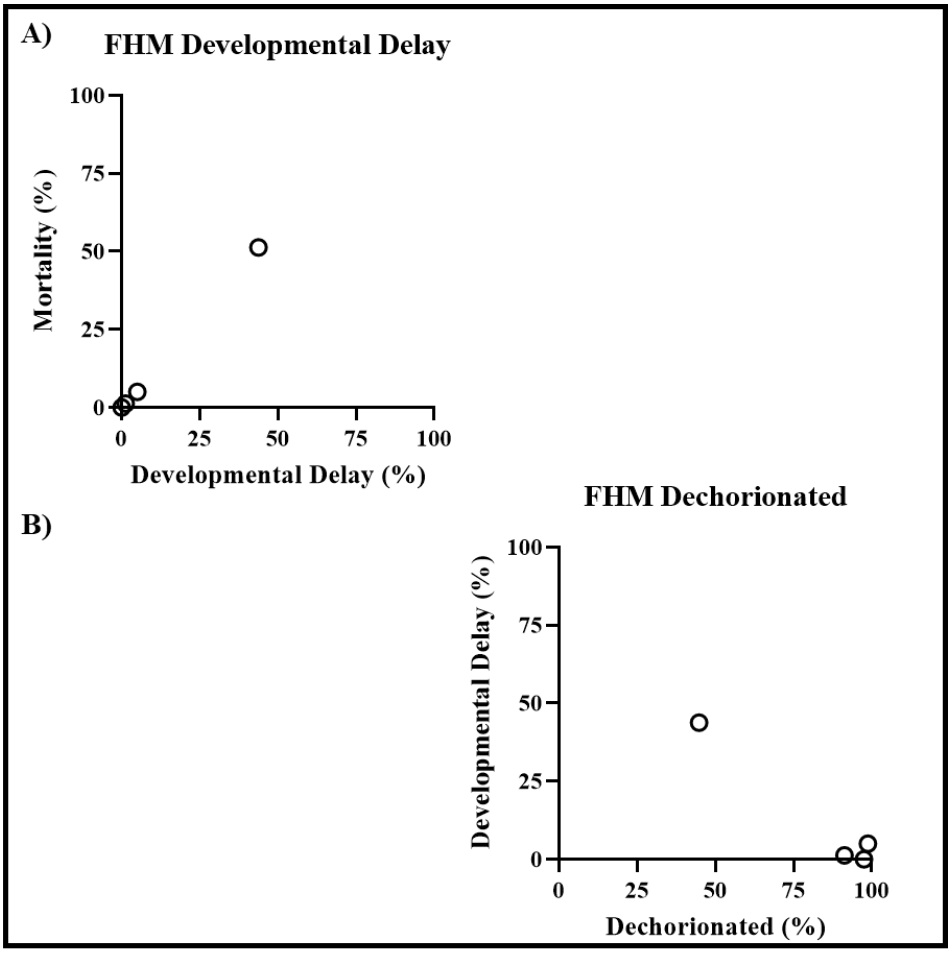

Figure 3-4. Association in the FHM Mixture Study: Developmental Toxicity. Association with the incidences of (A) developmental delay and cumulative mortality; and (B) natural dechorionation and developmental delay are displayed with values from four replicates in each plot from the FHM mixture study.

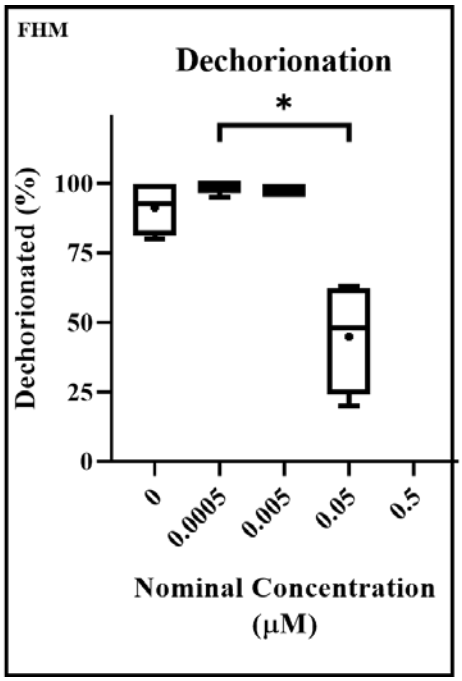

Figure 3-5. Dechorionation in the FHM Mixture Study. Percent of FHM embryos that dechorionated by Day 6 of the FHM mixture study is displayed. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. $p \leq 0.05$ (*)

Lethal and sublethal effects were observed in two different fish species. Mortality did not exceed 6% in the unexposed groups, in the nominal 0.0005 µM (Ultra Low) fathead minnow exposure group, or in either of the nominal 0.005 µM (Low) exposure groups (in zebrafish or fathead minnow studies). Developmental delay exceeded 5% in both of the nominal 0.05 µM (Medium) exposures (in zebrafish or fathead minnow studies) and in the nominal 0.5 µM (High) zebrafish exposure group. Assuming that mortality is related to the logarithm of concentration, the estimated LC_{50} was calculated to be 0.02 µM (nominal) in the ZF mixture study and an estimated LC_{50} was calculated to be 0.05 µM (nominal) in the FHM mixture study. Assuming that delayed development (gastrulation defects) is related to the logarithm of concentration, the estimated EC_{50} was calculated to be 0.07 µM (nominal) in the ZF mixture study. An estimated EC_{50} was calculated to be 0.04 µM (nominal) in FHM embryos that experienced delayed natural

dechorionation when exposed to a nine-drug mixture. No and Lowest Observable Adverse Effect Levels (NOAEL and LOAEL) were determined. The NOAELs for developmental progress were the nominal 0.05 µM (Medium) treatment in the ZF mixture study and the nominal 0.005 µM (Low) treatment in the FHM mixture study; the LOAELs were the nominal 0.5 µM (High) treatment in the ZF mixture study and the nominal 0.05 µM (Medium) treatment in the FHM mixture study. The NOAEL for dechorionation was greater than the nominal 0.05 µM (Medium) treatment in the FHM mixture study.

Muscle Abnormalities

The second category of observations assessed was myotoxicity, which included measuring the maximum velocity achieved in response to a touch stimulus and observing muscle fiber arrangements (birefringence). The touch assay was only performed with nontransgene-expressing zebrafish embryos on Day 2 but muscle fiber arrangements were assessed in the transgene-expressing ZF on Day 3 and in the dechorionated FHM embryos on Day 6. Zebrafish embryos were manually dechorionated prior to participating in the touch assay, which involved a gentle prod to the posterior end with a dissecting probe. Birefringence involved observing how light passed through muscle fibers using a polarized lens. Assessment of this endpoint also required embryos to be dechorionated.

The thigmotactic response in nontransgene-expressing zebrafish embryos was not significantly affected by exposure to varying concentrations of a mixture of all nine drugs (Appendix 2). Twenty-nine percent of the transgene-expressing zebrafish embryos exhibited disrupted muscle fiber patterns in the nominal 0.005 µM (Low) exposure group compared to just 2% in the unexposed embryos (Figure 3-6).

Eighty percent of FHM embryos in one replicate from the nominal 0.05 µM (Medium) exposure were not dechorionated by Day 6 when this assessment occurred; this percentage was 33% higher compared to the average response of the remaining three replicates (47%). Therefore, FHM birefringence results were analyzed including and excluding this replicate. In comparison to another abnormal dechorionation response (early dechorionation, not delayed dechorionation), an average early dechorionation incidence of 31% was observed in surviving zebrafish embryos (from the unexposed (23%) and the nominal 0.005 µM (Low) (39%) exposure groups).

FHM embryos treated with the nominal 0.05 µM (Medium) treatment exhibited significant disruption to the patterns of their muscle fibers compared to unexposed embryos (Figure 3-6). Four percent of unexposed FHM embryos exhibited abnormal patterning. Birefringence patterns of the FHM embryos in the nominal 0.05 µM (Medium) mixture treatment group were also significantly different from those in the nominal 0.0005 µM (Ultra Low) mixture exposure group. These incidences were associated with those of FHM dechorionation (four replicates) (Figure 3.7). When statistical analysis was performed with only three replicates from the nominal 0.05 µM (Medium) exposure group, there were no significant changes in the results from birefringence pattern abnormalities in the FHM embryos (data not shown).

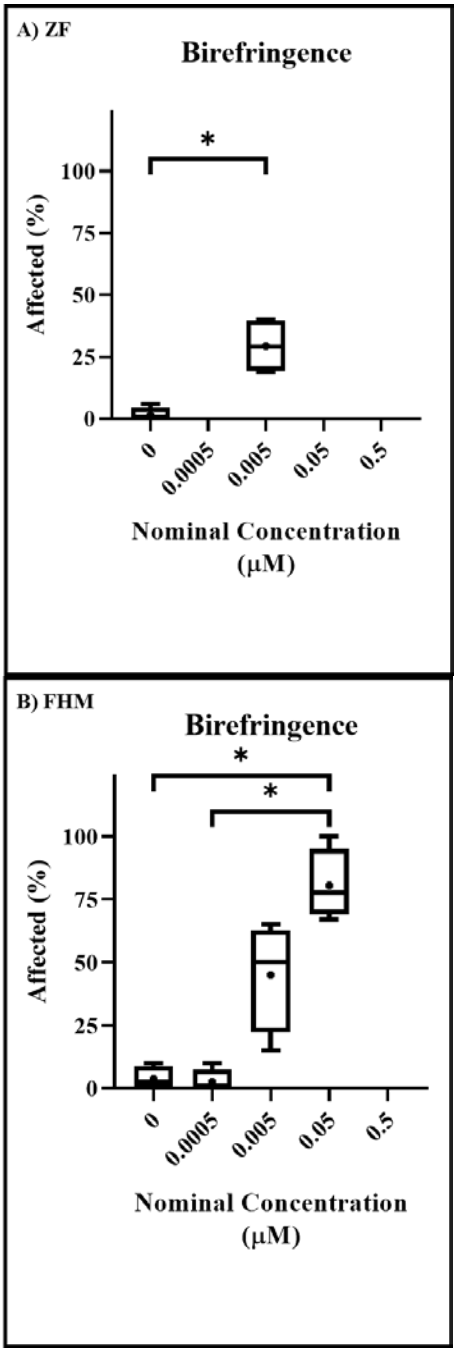

Figure 3.6. Abnormal Muscle Fiber Arrangement Using Birefringence in Mixture Studies. Percent of embryos with abnormal muscle fiber patterns from (A) ZF and (B) FHM mixture exposure studies is displayed. Only transgene-expressing zebrafish embryos were evaluated. No assessment of ZF embryos for this criterion was performed at the nominal 0.05 and 0.5 μM concentrations due to complete mortality. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25^{th} - 75^{th} percentiles), and whiskers are minimum and maximum values. Refer to Appendix 2 for corresponding morphology pictures. $p \leq 0.05$ (*)

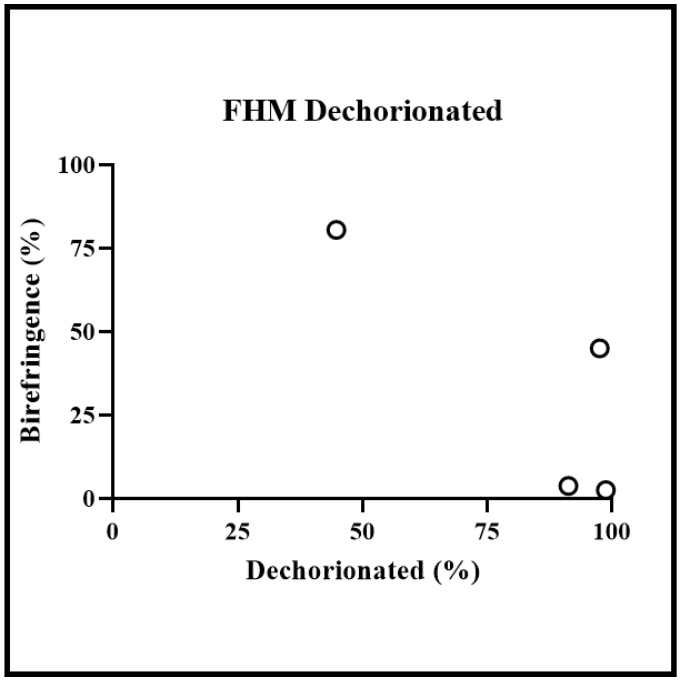

Figure 3-7. Association in the FHM Mixture Study: Muscle Abnormalities. Association with the incidences of dechorionation and abnormal muscle fiber pattern arrangement using birefringence from the FHM mixture study is displayed with values from four replicates.

No abnormal swimming speed response was observed in the exposed, nontransgene-expressing ZF embryos but muscle abnormalities were significantly observed in both species. Muscle fiber pattern abnormalities did not exceed 50% in the ZF embryos but assuming that muscle fiber pattern abnormalities are related to the logarithm of concentration, the estimated EC_{50} was calculated to be 0.007 µM (nominal) in the FHM mixture study. The NOAEL for disrupted birefringence was the nominal 0.005 µM (Low) treatment in the FHM mixture study; the LOAEL was the nominal 0.05 µM (Medium) treatment in the FHM mixture study.

Despite only scoring for the presence or absence of muscle fiber abnormalities, subjective observations noted more severe disruptions exhibited by the FHM embryos compared to those in the ZF mixture study, which corresponded with postmortem observations. Histopathological observations of hematoxylin and eosin (H & E)-stained zebrafish embryos did not support the *in vivo* conclusion of muscle damage. Preliminary pathology did corroborate *in vivo* observations of

unexposed and exposed H & E-stained FHM embryos. Microscopic damage including myocytic necrosis, increased presence of macrophages, edema, and disrupted muscle fiber arrangements was observed in FHM embryos exposed to the mixture of nine antilipidemic drugs (Figure 3-8).

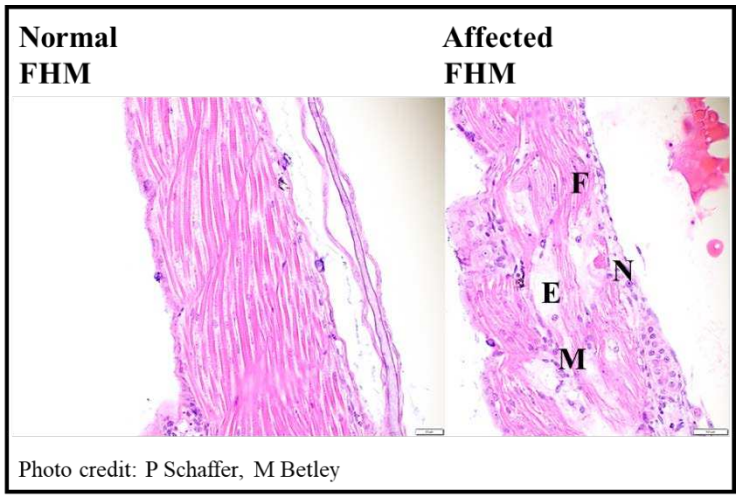

Figure 3-8. Presence of Myotoxicity from the FHM Mixture Study. H&E staining of FHM embryos after six days of exposures to a mixture of nine antilipidemic drugs. A portion of the posterior end of each embryo is shown. The presence of myotoxicity (necrosis-"N," edema-"E," more macrophages-"M," and disrupted muscle fiber pattern-"F") is shown at 400x.

Yolk Abnormalities

The third category of observations assessed was yolk abnormalities, which included the extent of yolk absorption and anterior to posterior (AP) axis determination. These two observations were assessed in transgene-expressing zebrafish embryos and fathead minnow embryos on Day 3 or 6, respectively. Yolk absorption was quantified by outlining the yolk area, including the yolk extension, with ImageJ software. Any deviation from a 180-degree AP axis was visually observed and evaluated as abnormal. Both observations required the embryos to be dechorionated and positioned laterally.

The average amount of yolk remaining in embryos at test termination was not significantly impacted in either mixture study (Appendix 2). However, the occurrence of AP axis

deformities in the zebrafish-treated embryos at the nominal 0.005 µM (Low) exposure level (27%) and in the fathead minnow embryos exposed to the nominal 0.05 µM (Medium) exposure level (55%) was significantly more frequent compared to incidences in all lower concentration groups and in the unexposed embryos (Figure 3-9). The incidence of AP axis deformities did not exceed more than 4% in the control groups of either study. Mild and severe abnormalities of the axis were observed in both studies, but this abnormality was only evaluated dichotomously. Assuming that altered AP axis is related to the logarithm of concentration, the estimated EC_{50} was calculated to be 0.04 µM (nominal) in the FHM mixture study. The NOAEL for AP axis deformities was the nominal 0.005 µM (Low) treatment in the FHM mixture study; the LOAEL was the nominal 0.05 µM (Medium) treatment in the FHM mixture study.

Due to the previously-described decrease in dechorionated FHM embryos on Day 6, this category was also analyzed with and without the replicate from the nominal 0.05 µM (Medium) exposure level included. When statistical analysis was performed with only three replicates from the nominal 0.05 µM (Medium) exposure group for yolk areas, the lack of significant findings did not change in the FHM embryos, but the results for AP axis abnormalities did. Significant AP axis deformities were observed between the same groups as when analyzed with four replicates but, different p values were calculated. The calculated p value between the nominal 0.05 µM (Medium) exposure level and the nominal 0.005 µM (Low) exposure level shifted from $p < 0.05$ to $p < 0.01$ when three replicates were included. A p value shift from $p < 0.01$ to $p < 0.001$ was also observed between the nominal 0.05 µM (Medium) exposure group and the nominal 0.0005 µM (Ultra Low) exposure group when one replicate was excluded from the analysis.

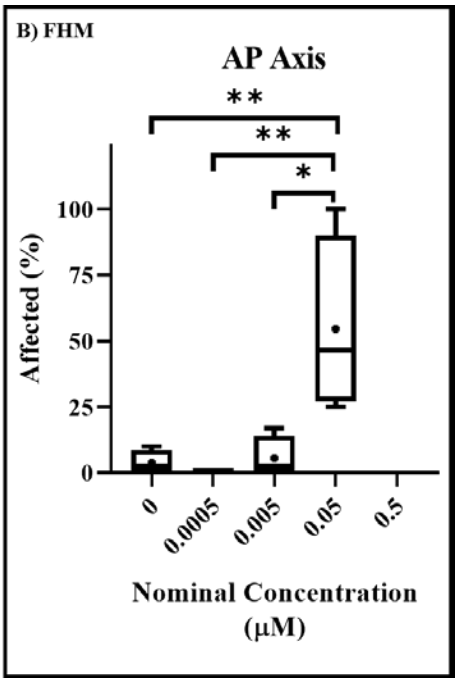

Figure 3-9. Altered Anterior-Posterior Axis Development in Mixture Studies. Percent of embryos with abnormal angles to their anterior-posterior axes from (A) ZF and (B) FHM mixture exposure studies is displayed. Only transgene-expressing zebrafish embryos were evaluated. No assessment of ZF embryos for this criterion was performed at the nominal 0.05 and 0.5 μM concentrations due to complete mortality. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 2 for corresponding morphology pictures. $p \leq 0.05$ (*), $p \leq 0.01$ (**)

Cardiovascular Abnormalities

The fourth and final category of observations assessed was cardiovascular abnormalities, which included vessel development, heart rate, and presence of edema and hemorrhage. All but the heart rate were assessed in dechorionated, transgene-expressing zebrafish embryos on Day 3 of the mixture study; all but the vessel development were assessed in dechorionated fathead minnow embryos on Day 6 of the mixture study. Intersegmental vessel (ISV) and subintestinal vein (SIV) development were evaluated with fluorescence. ISV development was scored abnormal when one or more was missing or was incompletely traversing the space between the dorsal aorta and the dorsal longitudinal anastomotic vessels. SIV development was subdivided into three categories: underdeveloped (SIV-under), overdeveloped (SIV-over), and missing

(SIV-missing). The sum of the three subcategories was statistically analyzed. The average heart rate was measured three times in an FHM embryo prior to anesthesia and subsequent observations. The presence of pericardial or yolk edema or hemorrhage were dichotomously evaluated but subjective severities and hemorrhage locations were observed; severe examples of this abnormality in each species were captured (Appendix 2); the most common location of hemorrhage in both species was the developing brain region.

Abnormal development of zebrafish intersegmental vessels in the nominal 0.005 µM (Low) exposure group (13% ± 0% standard deviation) was significantly more frequent compared to development in the unexposed (2% ± 3% standard deviation) (Figure 3-10). Subintestinal vein development in ZF embryos was not significantly affected (Appendix 2). The presence of pericardial edema was significantly higher in the nominal 0.005 µM (Low) exposure group compared to the unexposed group (Figure 3-11). Histopathological assessments were blindly evaluated for mild and severe pericardial effusion (Figure 3-12) but no significant differences were observed in ZF embryos after exposure to antilipidemic drugs (data not shown). The presence of edema around the yolk sac (yolk edema) observed in zebrafish was significantly greater in the embryos exposed to the nominal 0.005 µM (Low) mixture than in the unexposed embryos (Figure 3-13). The presence of hemorrhage recorded in exposed ZF embryos (0.005 µM (Low) exposure group) was greater compared to the frequency observed in Control embryos in the mixture study (Figure 3-14).

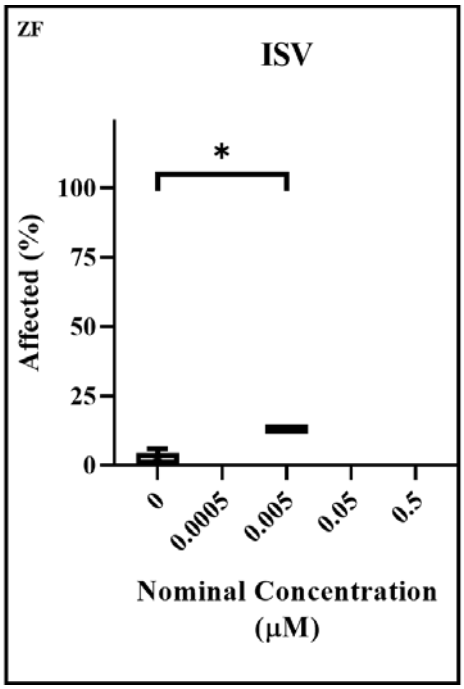

Figure 3-10. Abnormal Intersegmental Vessel Development in Mixture Studies. Percent of transgene-expressing embryos with abnormal ISV development from the ZF mixture exposure study is displayed. ISV development was scored abnormal when one or more was missing or was incompletely traversing the space between the dorsal aorta and the dorsal longitudinal anastomotic vessels. No assessment of ZF embryos for this criterion was performed at the nominal 0.05 and 0.5 μM concentrations due to complete mortality. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 2 for corresponding morphology pictures. $p \leq 0.05$ (*)

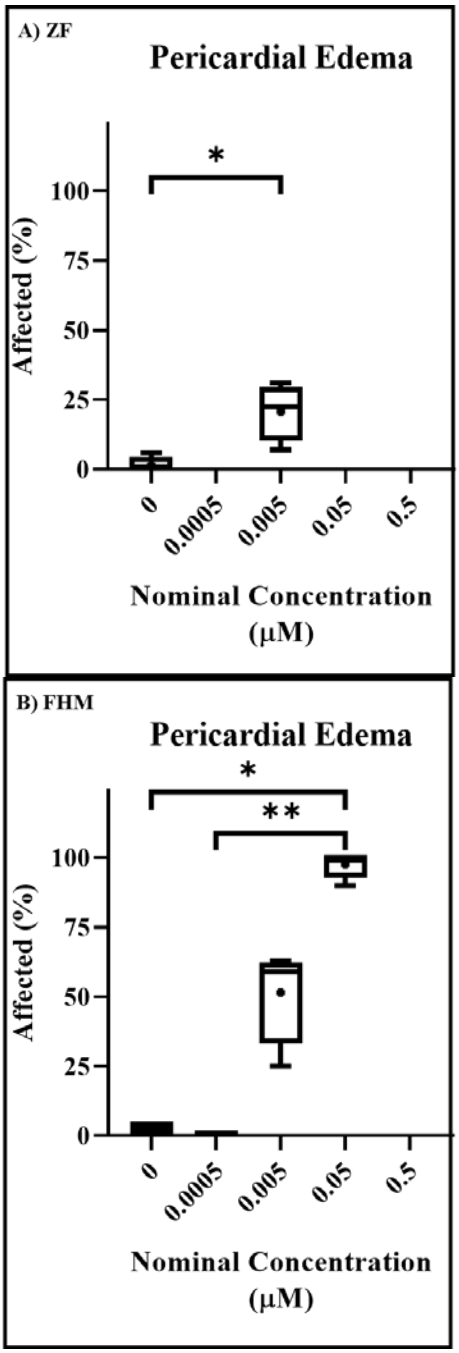

Figure 3-11. Presence of Pericardial Edema in Mixture Studies. Percent of embryos with pericardial edema from (A) ZF and (B) FHM mixture exposure studies is displayed. Only transgene-expressing zebrafish embryos were evaluated. No assessment of ZF embryos for this criterion was performed at the nominal 0.05 and 0.5 μM concentrations due to complete mortality. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 2 for corresponding morphology pictures. $p \leq 0.05$ (*), $p \leq 0.01$ (**)

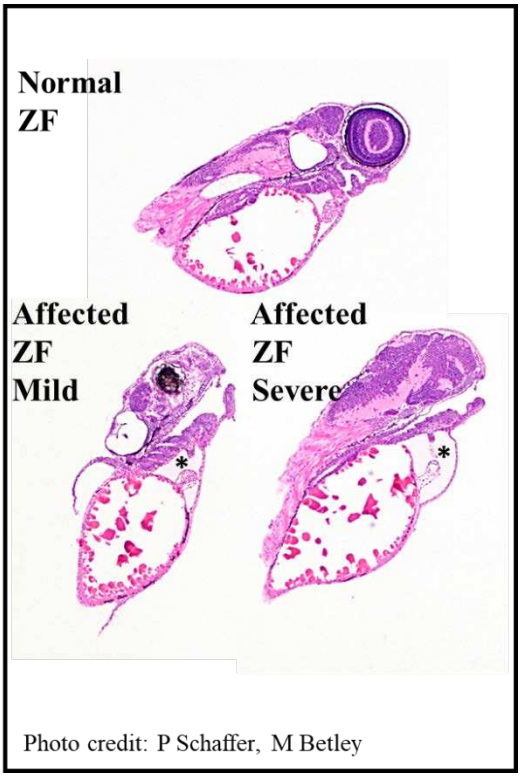

Figure 3-12. Presence of Pericardial Effusion in the ZF Mixture Study. H&E staining of ZF embryos after three days of exposure to a mixture of nine antilipidemic drugs. The anterior region of each embryo is oriented to the top-right. Nontransgene- and transgene-expressing embryos were analyzed. The presence of mild and severe pericardial effusion is shown ("*") at 400x.

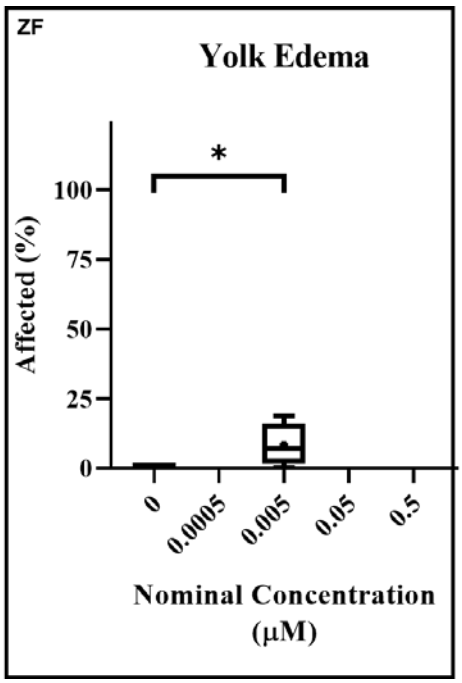

Figure 3-13. Presence of Yolk Edema in the ZF Mixture Study. Percent of transgene-expressing embryos with yolk edema from the ZF mixture exposure study is displayed. No assessment of ZF embryos for this criterion was performed at the nominal 0.05 and 0.5 μM concentrations due to complete mortality. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 2 for corresponding morphology pictures. $p \leq 0.05$ (*)

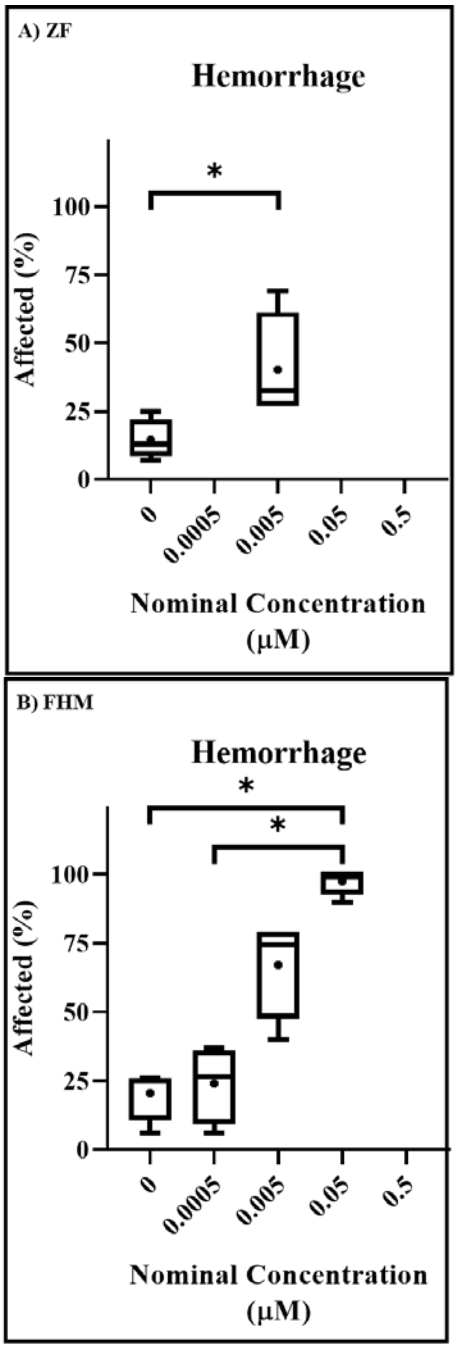

Figure 3-14. Presence of Hemorrhage in Mixture Studies. Percent of embryos with hemorrhage from (A) ZF and (B) FHM mixture exposure studies is displayed. Only transgene-expressing zebrafish embryos were evaluated. No assessment of ZF embryos for this criterion was performed at the nominal 0.05 and 0.5 µM concentrations due to complete mortality. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 2 for corresponding morphology pictures. $p \leq 0.05$ (*)

Fathead minnow embryos in the nominal 0.05 µM (Medium) exposure group appeared the most affected due to the presence of several cardiovascular abnormalities and visible opacity to cells beginning to transpire at test termination. With only one beat difference, the average heart rates in the FHM embryos not exposed to the mixture of drugs (167 beats per minute (bpm)) and those exposed in the nominal 0.0005 µM (Ultra Low) (166 bpm) were not significantly different (Figure 3-15). However, the heart rates of FHM embryos exposed to the two higher treatment levels (0.005 µM: 127 bpm, 0.05 µM: 115 bpm) were significantly different from these two groups treated at lower concentrations. The presence of pericardial edema in FHM embryos were also significantly different from the frequencies observed in the nominal 0.0005 µM (Ultra Low) and nominal 0.05 µM (Medium) exposure groups. Three percent of unexposed FHM embryos exhibited pericardial edema. These pericardial edema incidences were associated with those of FHM dechorionation (four replicates) (Figure 3-16). Severe pericardial edema was corroborated histopathologically during preliminary observations but the full analysis of FHM embryos is currently pending (Figure 3-17). The presence of yolk edema was not significantly affected when fathead minnow embryos were exposed to a mixture of nine antilipidemic drugs. These yolk edema incidences were associated with those of FHM dechorionation (four replicates) (Figure 3-18). However, when statistical analysis was performed with only three replicates from the nominal 0.05 µM (Medium) exposure group for yolk edema, the results changed (Figure 3-19). The incidence of yolk edema observed in the FHM embryos in the nominal 0.05 µM (Medium) exposure group (53%, 3 replicates) was now significantly different from the incidences recorded in the Control (0%) and nominal 0.0005 µM (Ultra Low) (0%) exposure groups. Additionally, the incidence in the nominal 0.005 µM (Low) (43%) exposure group was significantly different from the incidences calculated in the nominal 0.0005

µM (Ultra Low) and Control groups. The presence of hemorrhage in the nominal 0.05 µM (Medium) FHM exposure group (98%) was greater compared to the frequencies observed in the nominal 0.0005 µM (Ultra Low) (24%) and Control (21%) groups in the FHM mixture study. These hemorrhage incidences were associated with those of FHM dechorionation (four replicates) (Figure 3-20).

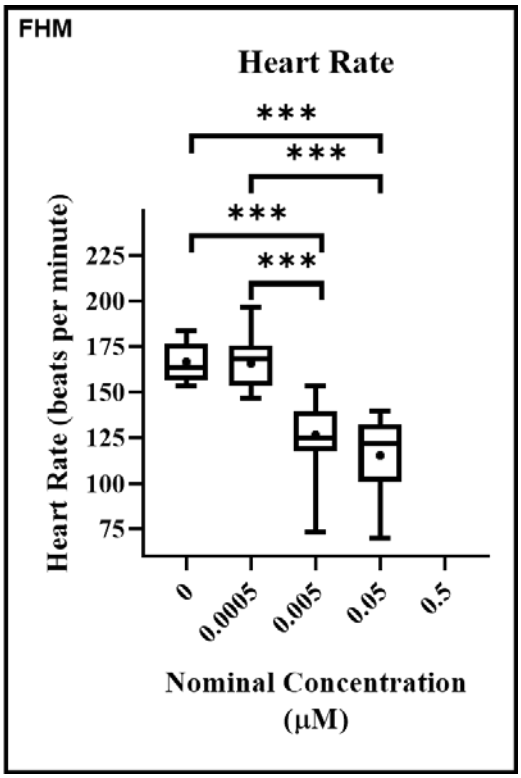

Figure 3-15. Mean Heart Rate in the FHM Mixture Study. Heartrates of embryos from the FHM mixture exposure study are displayed. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. $p \leq 0.001$ (***)

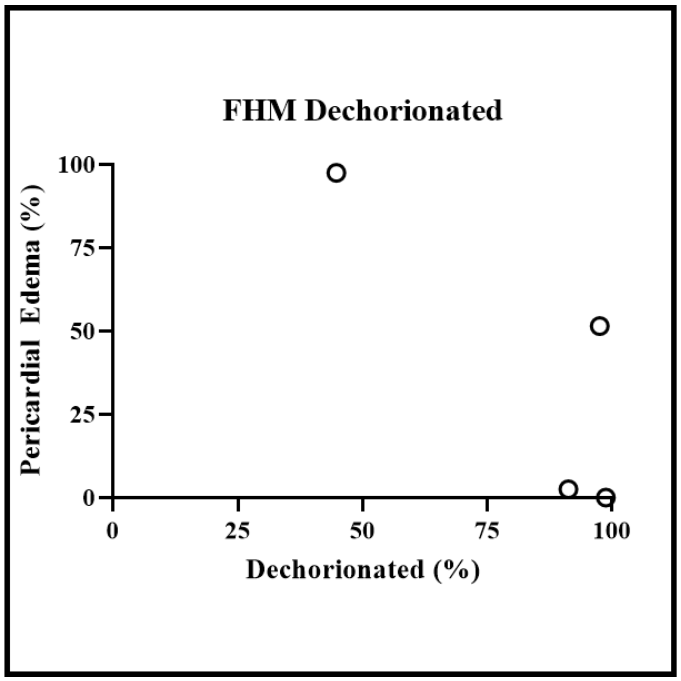

Figure 3-16. Association in the FHM Mixture Study: Cardiovascular Abnormalities. Association with the incidences of dechorionation and the presence of pericardial edema from the FHM mixture study is displayed with values from four replicates.

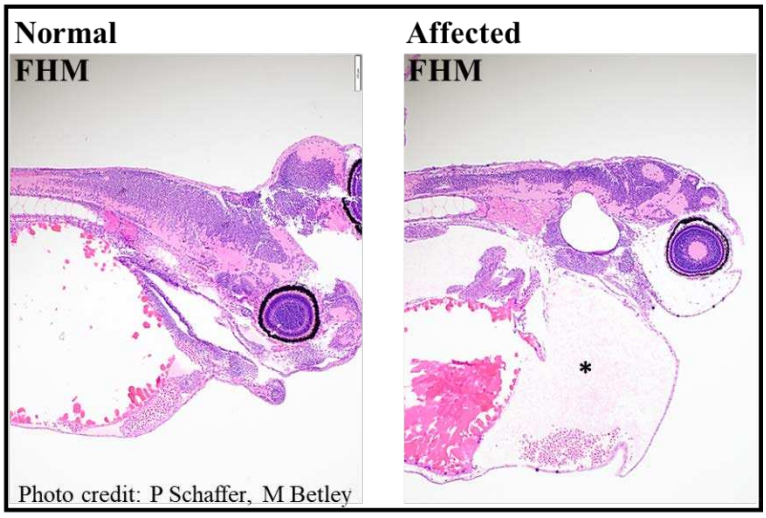

Figure 3-17. Presence of Pericardial Effusion in the FHM Mixture Study. H&E staining of FHM embryos after six days of exposures to a mixture of nine antilipidemic drugs. The anterior region of each embryo is oriented to the right. The presence of pericardial effusion is shown ("*") at 400x.

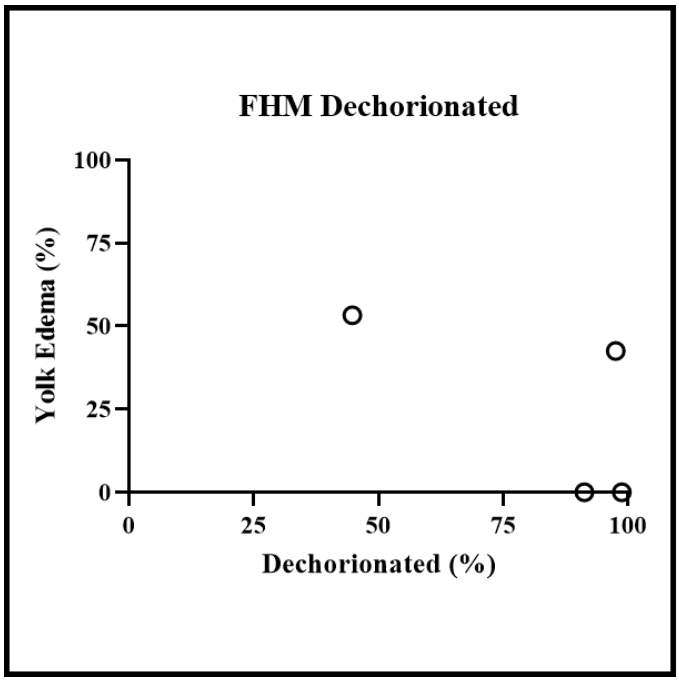

Figure 3-18. Association in the FHM Mixture Study: Cardiovascular Abnormalities. Association with the incidences of dechorionation and the presence of yolk edema from the FHM mixture study is displayed with values from four replicates.

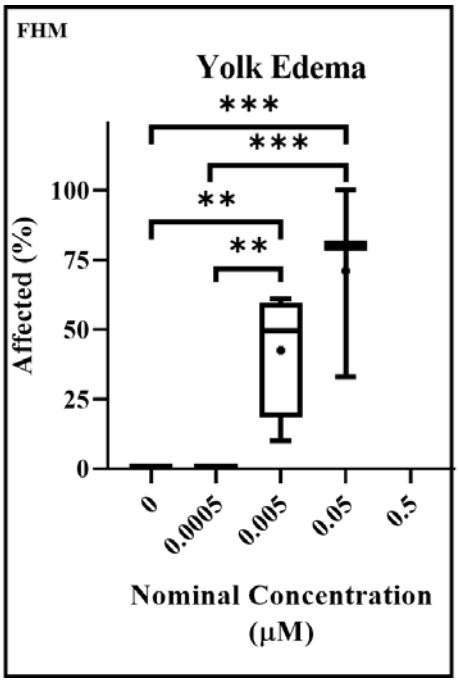

Figure 3-19. Presence of Yolk Edema in the FHM Mixture Study. Percent of embryos with yolk edema from the FHM mixture exposure study is displayed. Statistical analysis was performed with only three replicates in the Medium (0.05 µM) exposure group. See text for details. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 2 for corresponding morphology pictures. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***)

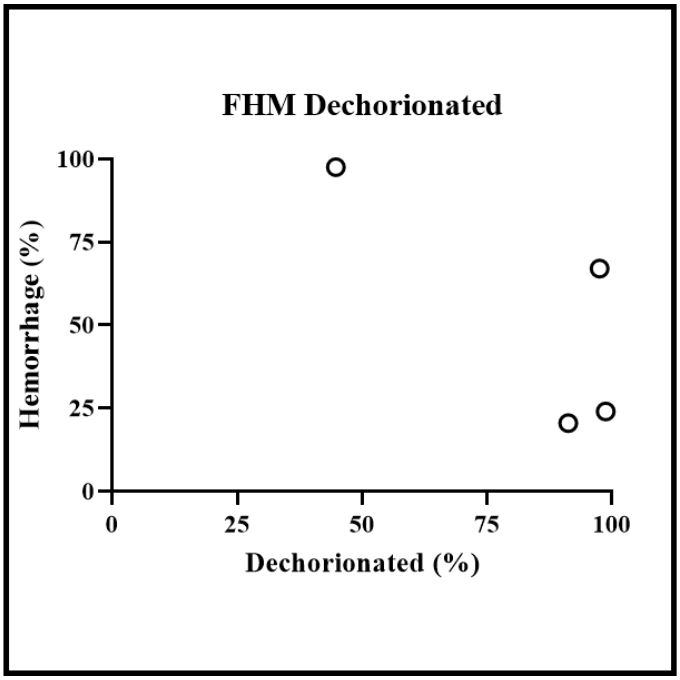

Figure 3-20. Association in the FHM Mixture Study: Cardiovascular Abnormalities. Association with the incidences of dechorionation and the presence of hemorrhage from the FHM mixture study is displayed with values from four replicates.

Significant cardiovascular effects were observed in zebrafish and fathead minnow embryos exposed to a mixture of nine antilipidemic drugs at nominal concentrations as low as 0.005 µM relative to unexposed embryos. The NOAEL for pericardial edema and hemorrhage was the nominal 0.005 µM (Low) treatment in the FHM mixture study; the LOAEL was the nominal 0.05 µM (Medium) treatment in the FHM mixture study (for both observations). The NOAEL for heart rate was the nominal 0.0005 µM (Ultra Low) treatment in the FHM mixture study; the LOAEL was the nominal 0.005 µM (Low) treatment in the FHM mixture study. Assuming that hemorrhage is related to the logarithm of concentration, the estimated EC_{50} was calculated to be 0.002 µM (nominal) in the FHM mixture study. An estimated EC_{50} was calculated to be 0.04 µM (nominal) FHM embryos that experienced pericardial edema when exposed to the mixture study.

Discussion

Fish embryos were successfully exposed to mixtures of nine antilipidemic drugs. Of the 15 different developmental effects assessed in ZF embryos and the 10 characterized in FHM embryos, eight observations were assessed for both species. Vessel development and maximum swimming speed were not assessed in FHM. Heart rate was not assessed in ZF embryos. The amount of yolk remaining at test termination was the only biological characteristic evaluated in both species that did not yield significant results in either species.

Of the seven other developmental outcomes assessed in both species, significant effects were detected except for two observations: yolk edema and dechorionation. The presence of yolk edema was not significantly increased in FHM embryos as a result of the mixture exposures but was when ZF embryos were exposed to a mixture of nine drugs. When a single replicate from the nominal 0.05 µM (Medium) exposure group was excluded from analysis of the FHM embryo results due to the low number of embryos that were dechorionated by six days, yolk edema incidences significantly increased in the nominal 0.005 µM (Low) and 0.05 µM (Medium) exposure groups compared to the unexposed group of embryos. An average of 53% of the embryos in the remaining three replicates dechorionated and the incidence of yolk edema increased from 53 ± 45% up to 71 ± 34% (mean ± standard deviation). Subjective observations of yolk edema location were noted in addition to edema size. Dechorionation was assessed by the natural progression in FHM embryos and by the number that dechorionated early in zebrafish embryos. Early manual dechorionation of zebrafish embryos (< 8 hpf) previously resulted in decreased survival rates (Henn & Braunbeck, 2011) and thus may pose a risk to developing embryos. This process was significantly delayed in FHM embryos exposed to a mixture of nine antilipidemic drugs at a nominal exposure concentration of 0.05 µM (Medium) compared to a

very low exposure (0.0005 µM nominal). This rate was associated with the high occurrence of hemorrhage and pericardial edema at this exposure level. Early dechorionation was not significantly affected in zebrafish-exposed embryos, but embryos should be allowed to progress naturally in a future study to more accurately compare to the observed results from the fathead minnow mixture study.

Of the five remaining toxic effects observed in both species, ZF embryos were more sensitive than FHM embryos. Interestingly though, despite scoring dichotomously, hemorrhage, yolk edema, muscle fiber patterning, and pericardial edema appeared more severe in FHM embryos. Pericardial edema was associated with delayed dechorionation observed in FHM embryos; therefore, based on the high incidence of these abnormalities and their association to dechorionation, it is presumed that fish health was sufficiently compromised as to prevent dechorionation at the appropriate time. Furthermore, due to the increased incidence of these sublethal abnormalities in the FHM embryos exposed to the nominal 0.05 µM (Medium) treatment, and the observed cell opacity occurring at test termination, mortality seemed imminent.

The more extensive FHM abnormalities could also be a result of the increased exposure time of FHM embryos (which was extended three additional days compared to the ZF mixture exposure). However, both studies terminated approximately one day after natural hatching should have occurred. Additionally, the speed of ZF development is faster compared than that of FHM embryos (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; USEPA, 1996). This interspecies variation is consistent with previous work in which FHM and ZF embryos responded to single drug exposures with different sensitivities (Steele et al., 2018). Two differences to note between Steele et al.'s (2018) work and the studies presented here are the

exposure time and the starting embryo ages. In Steele et al.'s (2018) study, the exposure times (for ZF and FHM) were 96 hours (in compliance with standard testing methods); ZF were 4-6 hpf and FHM were within 24 hours post hatch at test initiation. In the studies presented in this chapter, the exposure times varied so that similar developmental stages would be achieved by termination, but starting ages were similar (blastula stage of development or younger for both species).

Another factor that could contribute to interspecies variation is the presence of the chorion. Manual dechorionation was initiated in ZF embryos to allow thigmotaxis evaluations but manual dechorionation did not take place in FHM embryos due to the limited perivitelline space, thicker chorionic membrane, and high potential of damage to an embryo with a manual dechorionation process. The continued presence of the chorions in the FHM embryos may have provided extra protection, stabilized potential unfavorable changes in gases, liquids, or temperatures, and supplied micronutrients for the embryo in the perivitelline fluid (Laale, 1980). These advantages may have resulted in increased zebrafish sensitivity but assessing how the FHM chorion functions in the presence of contaminants and how it may impact the severity of observed abnormalities requires further investigation.

In both FHM and ZF mixture studies, aside from the control, two of three exposure concentrations were used in common: Low (nominal 0.005 µM of each drug) and Medium (nominal 0.05 µM of each drug). Because of the complete mortality observed in the Medium and High (0.5 µM) exposure groups in the ZF mixture study, an Ultra Low treatment level (0.0005 µM) was assessed instead of a High level in the FHM mixture study. FHM were less sensitive to the nominal 0.05 µM (Medium) treatment than ZF, with only 51% cumulative mortality. The observed FHM mortality in the Medium treatment group was associated with embryos that were

developmentally delayed (44 ± 6%; mean ± standard deviation). Morphologically, those FHM embryos failed to make much developmental progress between Days 1 and 2. An association between developmental delay and dechorionation was observed. Interestingly, failure to complete gastrulation in exposed ZF embryos was also associated with ZF mortality in the nominal 0.05 µM (Medium) treatment group. Lewis Wolpert, a famous developmental biologist, stated that gastrulation is the most important time in one's life, not birth, marriage, or death (Wolpert, 1991). Embryos that did not establish three germ layers successfully, develop body axes, and have cells efficiently move into new positions when exposed to nine antilipidemic drugs, did not survive in these studies.

During the time of gastrulation in the FHM mixture study, embryos remained at room temperature (23 °C) for an additional two hours after exposure began. No previous research was found that assessed impacts on development of this brief initial thermal deviation although several studies examined rearing temperatures (Brian, Beresford, Margiotta-Casaluci, & Sumpter, 2011; Georgakopoulou et al., 2007; Schaefer & Ryan, 2006; Sfakianakis, Leris, Laggis, & Kentouri, 2011). ZFIN, the zebrafish model organism online database, reported that ZF embryos developed normally within 3-5 °C of the ideal temperature (28.5 °C). Regardless of development at temperatures deviating from this optimum (25 °C or 33 °C), ZF embryos generally exhibited a linear response regarding time and developmental stage (Kimmel et al., 1995). For example, the shield stage (during gastrulation) was achieved about 6 hpf when incubated at the ideal 28.5 °C, but this stage would be reached about one hour sooner at 33 °C or approximately two hours later at 25 °C. As the embryo ages and is reared continuously at a non-ideal temperature, the differences in time to reach each developmental stage increase. Morphology of a 72 hpf embryo is observed at about 65 hpf or at nearly 90 hpf at 33 °C or

25 °C, respectively (Kimmel et al. 1995). This temperature effect was even suggested as a tool for researchers to standardize embryonic ages for experiments (Kimmel et al., 1995; ZFIN, 2019). Nonetheless, ZF embryos raised during embryogenesis at non-ideal temperatures still completed gastrulation. The FHM morphology in the Control exposure group in this study at Day 1 was similar to previously reported morphology at 24-30 hpf (USEPA, 1996) and a low incidence of sublethal effects was observed. A dose-dependent response was observed for disrupted birefringence pattern, hemorrhage, pericardial edema, and heart rate.

Sublethal cardiovascular effects including pericardial edema were observed in a dose-dependent response in the FHM and ZF embryos exposed to the 0.005 µM (Low) mixture supporting previous research that demonstrated both FHM and ZF embryos were capable of developing embryonic abnormalities when exposed to toxicants (Jeffries et al., 2015). Compared to previous observations of pericardial edema increasing in morphological severity as contaminant concentration increased (Jeffries et al., 2015), more noticeable differences were observed in the severity between the two tested species, although not with increasing concentrations. However, these studies used dichotomous scoring to avoid potential inconsistent subjective scoring of mild versus severe edema. Thus, this variable interspecies response could not be statistically assessed.

Histopathology was performed post-mortem for more accurate assessment of adverse effects. No significant differences between ZF groups was observed when comparing mild, severe, or total pericardial edema. The FHM analyses are currently pending. Two possible explanations for a lack of statistically significant pathological results but significant *in vivo* observations in the ZF samples were related to the embryo position and possibly due to fixation. ZF embryos were aligned in the embedding cassettes as best as possible but not every embryo

was correctly positioned for accurate analysis: 43 ± 10% (mean ± standard deviation) and 58 ± 14% (Control and Low exposure groups, respectively) (data not shown). It is possible that this led to an underestimation of the percentage of affected embryos. Embryos were fixed and remained in Bouin's solution until analysis, which may have led to brittle tissue or tissue shrinkage (Bultitude et al., 2011; Chatterjee, 2014; Kahyaoğlu & Gökçimen, 2017). However, Bouin's is an excellent preservative for certain soft and fragile human tissues, such as testicular biopsies (Bultitude et al., 2011) and it is less likely that fixation affected the results as much as positioning.

Heart and blood vessels are the first organs exposed to toxicants during embryogenesis (Han et al., 2018), and the heart is the first functional organ to develop during organogenesis (Singleman & Holtzman, 2012). The zebrafish heart, as an elongated tube, contracts at 26 hpf (Kimmel et al., 1995) and embryos do not rely on this organ for the exclusive delivery of oxygen until the larval stage of development (Singleman & Holtzman, 2012). Without a functional cardiovascular system, embryos will not survive to adulthood. However, ZF embryos are able to obtain sufficient oxygen by diffusion through five days of development, permitting mutants with lethal heart defects to survive at least this long (Gonzalez-Rosa, Burns, & Burns, 2017). Hemorrhage, decreased FHM heart rates, pericardial edema, and abnormal development of ZF vessels (ISVs), were observed in the living ZF and FHM embryos exposed to a mixture of antilipidemic drugs. Such observation is not possible in other vertebrate models, such as living mouse embryos, without dissection at single timepoints (Singleman & Holtzman, 2012). Toxicant-related decreased heart rates were previously recorded in ZF embryos up to 4 dpf (Han et al., 2018). These sublethal observations in embryos are useful for scientists to better understand and predict toxic responses when larval observations may be too late (Jeffries et al.,

2015). Future work could focus on tracking how ZF regenerate (Itou, Kawakami, Burgoyne, & Kawakami, 2012) and possibly recover from these sublethal, embryonic, cardiovascular, adverse responses. Despite the lack of overdevelopment of SIVs in ZF embryos in this mixture study, this work could be performed specifically with statins because statins have also been shown to stimulate survival and angiogenesis (Eichel et al., 2010).

If cardiovascular abnormalities such as pericardial edema were to develop in exposed embryos of other fish species, including salmonids, they may suffer further complications as they grow. As salmonids reached sexual maturity, they developed coronary arteriosclerosis. More than 95% of surveyed salmonids experienced lesions and these lesions increased in size and frequency as the fish grew (Saunders et al., 1992). Up to 50% occlusion of the coronary artery was measured in up to 85% of the length of this artery (Saunders, Farrell, & Knox, 1992). Therefore, abnormal cardiovascular function (i.e., decreased heart rates, presence of pericardial edema, etc.) resulting from exposure to a mixture of contaminants during embryonic development may compound the overall health of the developing fish, and if they naturally develop coronary arteriosclerosis, their ability to adapt, survive, and continue to grow may be compromised. However, further study would be required to investigate the potential confounding influence of diet, endocrinology, and vascular damage (Saunders et al., 1992) with exposure to antilipidemic drugs on growing fish.

The lack of significant effects on ZF embryos fleeing a touch stimulus when exposed to a low exposure level of a mixture of antilipidemic drugs was unexpected in view of muscle damage observed via birefringence. Perhaps the number of embryos assessed was too few, or this exposure concentration did not affect swimming speeds. One other potential explanation was the age of the ZF embryos. Individual ZF muscle myotomes begin contracting at approximately 18

hpf and gradual coordination with more myotomes occurs as time progresses. When a ZF embryo is less than 36 hpf, spontaneous contractions decrease and ZF embryos can reflexively respond to a stimulus (Kimmel et al., 1995). Our touch response was assessed when ZF embryos were approximately 48 hpf; this was attempted in FHM method development at 6 dpf but abandoned due to restlessness (unpublished observations). Perhaps the embryos were too young and required more development time. A similar approach, but with a vibrational stimulus, was successfully performed in 12-dpf FHM embryos (McGee et al., 2009) and initially developed with 5-30-dpf ZF embryos (H. Schoenfuss, personal communication, December 29, 2017). Phototaxis was also studied previously in ZF embryos and summarized by Steele et al. (2018). Because response to a stimulus is critical at any age for fish to avoid predators or other environmental threats, subsequent work to understand the development of this response in embryos in toxicant-exposed waters would be worthwhile.

A previous review of studies with exposures to clofibrate, a fibrate no longer prescribed to humans, or its metabolite, clofibrinic acid, identified an invertebrate, *Daphnia magna,* as the most sensitive organism with an EC_{10} of 8.4 µg/L based on reproduction. A zebrafish study reported reproductive effects and a fathead minnow study reported spermatogenesis effects at clofibrate concentrations equal to or greater than 100x the effective concentration observed in *D. magna* (Brausch, Connors, Brooks, & Rand, 2012). This EC_{10} (0.03 µM clofibrate) is within the nominal 0.005 µM (Low) and 0.05 µM (Medium) exposure groups from two mixture studies in this work. Therefore, this research contributed to the studied sublethal effects from antilipidemic exposure(s) because the researchers also did not observe significance in the zebrafish touch assay performed near this EC_{10}. An additional mixture exposure to effluent containing 662 ng GEM/L and six other common pharmaceuticals did not identify any significant lethal or sublethal effects

during the FHM early life stage test (Parrott & Bennie, 2009). However, a small but significant portion of offspring experienced deformities; spinal deformities and edema (yolk and pericardial) were the most commonly observed (Parrott & Bennie, 2009).

Additional beneficial future work would include transcriptomics, which could provide insight into genes that mediate the effects of toxic exposures (Qian, Ba, Zhuang, & Zhong, 2014; van Delft et al., 2012). Gene expression analyses elucidated a toxic molecular response to what was previously perceived as non-toxic effluent (based on survival) (Jeffries et al., 2015). Exposure of adult FHM to a ten-compound mixture containing 1.2 µg GEM/L also led to significant changes in gene expression (Zenobio, Sanchez, Archuleta, & Sepulveda, 2014). Additional molecular events may be discovered with RNA sequencing (Han, Gao, Muegge, Zhang, & Zhou, 2015; Li, Liu, Tang, Yang, & Meng, 2017).

Mechanisms related to embryonic metabolism or defense may also be better understood and may support morphological observations. The tissue expression pattern of organic anion transporters (OATs) in adult ZF (intestine, kidney, heart, and skeletal muscle) (Mihaljevic, Popovic, Zaja, & Smital, 2016) are conserved in FHM as well (transporters are found across the Bilateria clade and one subfamily of OATs is found in teleost fish) (Hagenbuch & Meier, 2004; Popovic et al., 2013). These transporters have been proposed as crucial in the defense of drug exposure (Mihaljevic et al., 2016). The embryonic transcriptome is highly active (Kimmel et al., 1995). Also, several orthologs are ubiquitously expressed in zebrafish tissue (Popovic, Zaja, Fent, & Smital, 2014). The zebrafish genome includes orthologous genes to Oatp1 transporters which are solute carrier transport proteins. Specifically, Oatp1d1 (also called slco1d1) transfers fibrates, statins, and other xenobiotics across cell membranes in adult zebrafish (Popovic et al., 2013; Popovic et al., 2014). Oatp1d1 is known to be expressed in zebrafish embryos (as early as

16 hpf) in the yolk syncytial layer, myotomes, pectoral fin, and developing eye structures (Thisse and Thisse, ZFIN, 2004). Therefore, it is assumed that antilipidemic drugs may in part exert their effects by interacting with transporters. This will activate signal transduction mechanisms that alter gene expression and lead to abnormal phenotypes.

Tissue concentration measurements for a mixture exposure would clarify a potential mode of action (Schultz et al., 2011) and how drugs may move through a food chain. GEM was detected in fish liver collected from a site downstream of a wastewater treatment plant (Ramirez et al., 2009). At higher, non-environmentally-relevant concentrations, nutritional, long chain, omega-3 fatty acids were reduced when juvenile female rainbow trout (*Onchorynchus mykiss*) were injected with 100 mg GEM/L five times over the course of 15 days (Prindiville, Mennigen, Zamora, Moon, & Weber, 2011). Uptake of drug(s) from an antilipidemic mixture has not been identified in aquatic organisms to the best of the author's knowledge.

Finally, consideration of transgenerational effects (Parrott & Bennie, 2009) is environmentally-relevant given fish may live their lives in drug-exposed environments. An influential factor for possible effects would be the composition of each effluent but additional relevant, potentially more sensitive, effects may be discovered such as decreased adult FHM reproduction, which was more sensitive than early embryogenesis observations with exposure to a perfluorinated compound (Ankley et al., 2005).

These two mixture studies conducted with fathead minnow and zebrafish embryos exposed to a nine-drug mixture, highlights interspecies response variation and identified sensitive sublethal criteria that can be used to screen for toxicity. Observations were more specific than some described in standard testing methods (EPA, 2002; OECD, 1998; OECD, 2013). For example, abnormal body form was evaluated more specifically as AP axis deviations

from 180 degrees in the mixture studies; four observations were recommended (coagulation, asystole, lack of somites, and lack of tail bud detachment) whereas these studies screened several more observations. Also, this project evaluated more than the minimum three replicates and more than the recommended number (n = 15) of embryos.

Nonetheless, the observed significant abnormalities occurred at nominal concentrations 10 to 1,000 times the environmentally-relevant concentrations previously reported. The FHM Ultra Low (0.0005 µM) group exposed to the nine antilipidemic drug mixture was the lowest tested concentration and was closest to concentrations measured in the environment (Table 2.1). The target concentrations for each of the nine drugs were 0.15 times up to 210 times greater than the ERC for eight of the nine drugs; the ERC for rosuvastatin was 1.25 times greater than the target Ultra Low concentration. The ERCs for seven of the nine drugs were observed effluent concentrations. Because pitavastatin was not recorded in effluent previously, a concentration similar to other statins was selected. Since the range of gemfibrozil concentrations in effluent varied from 2 ng GEM/L up to 19.4 µg GEM/L (Table S-7) and the average of this minimum and maximum is 9.7 µg GEM/L which was higher than most observed surface water concentrations, the maximum observed surface water concentration in the United States was selected. All concentrations were selected to represent the upper bound of an exposure detected in the environment and to evaluate if study exposure concentrations near these upper bounds exhibited adverse effects on fish embryogenesis. No significant adverse lethal or sublethal effects were detected at these concentrations. Although it is unlikely that all nine of the studied antilipidemic drugs will exist in nature concurrently, the significant adverse responses demonstrate embryonic fish are sensitive to aqueous pharmaceutical exposure. Surprisingly, the areas of the lipid-rich

yolk were not significantly affected when exposed to a mixture of nine antilipidemic drugs, but other effects such as altered AP axes were observed.

CHAPTER 4

INDIVIDUAL DRUG STUDIES

Studies with each of the nine antilipidemic drugs were performed using zebrafish embryos. Zebrafish (*Danio rerio*, ZF) were exposed to two nominal exposure levels (ERC Low and Very High (nominal 1 µM)) for 72 hours beginning with blastulating embryos four hours post fertilization (hpf) or younger. The ERC Low treatment group was targeted to be 10 times the observed environmental concentration from Table 2-2. Because this was drug-specific, a categorical value of < 0.003 µM was displayed on all plots. All nominal ERC Low values were below this value. These individual studies investigated if embryos exposed to a single drug at an environmentally-relevant concentration exhibited lethal or sublethal effects. Fifteen observations (lethal and sublethal) were evaluated in each individual drug study.

Water chemistry values for all individual studies ranged from pH 7.4 up to 8.1, dissolved oxygen never fell below 7.6 mg/L, and testing temperatures were within 2 °C of the target temperature (28 °C) once incubation began after the tests were initiated at room temperature (Appendix 4). Reported temperatures were taken while embryos were incubating and prior to relocation to temporary incubators; if temperatures were taken after the move, then they were not included in the summary table (Appendix 4).

Developmental Toxicity

The first category of observations assessed was developmental toxicity, which included cumulative mortality, developmental progress, and dechorionation. This category was assessed in both transgene- and nontransgene-expressing embryos. Embryos must have had a detectable heartbeat (if the heart had formed) or translucent cellular appearance to be evaluated as alive.

Mortality was evaluated daily, and cumulative mortality determined. Developmental progress was evaluated on Day 1. If an embryo had an abnormal shape or volume of yolk, an unhealthy yolk extension, or lack of developmental progress (i.e., somites present, optic vesicle visible, etc.), the embryo was evaluated as exhibiting gastrulation defects. Finally, prior to forced dechorionation on Day 2, the number of embryos that naturally dechorionated early was recorded. The outcome of these studies demonstrated embryonic exposure to FENO, GEM, ATO, FLUV, PRAV, and ROS did not exhibit any developmental toxicity (Appendix 3). Representative morphology corresponding to significant findings from the LOV, PIT, and SIM studies for this category were captured (Appendix 3).

Complete cumulative mortality was observed in embryos exposed to either LOV or SIM in the nominal 1 µM (Very High) treatment (Figure 4-1). Cumulative mortality was not significantly impacted with embryos exposed to any of the remaining seven individual drugs (Appendix 3). Mortality did not exceed 3% in the Control or ERC Low groups of either study and most mortality in either study occurred by Day 2. A statistical difference was observed in LOV-exposed embryos between the nominal 2.5 x 10^{-3} µM (LOV ERC Low) and the nominal 1 µM (Very High) exposure groups. In the SIM study, mortality of embryos was significantly different in the nominal 1 µM (Very High) exposure group compared to the Control and 2.4 x 10^{-5} µM (SIM ERC Low) exposure groups. Mortality was associated with the incidence of gastrulation defects in each of these two studies (Figure 4-2).

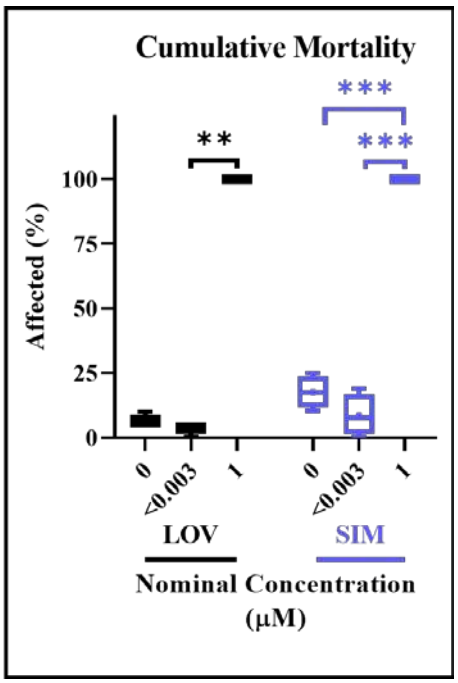

Figure 4-1. Cumulative Mortality in Individual Studies. Percent of embryos that died (cumulative mortality) from the individual drug studies is displayed. Transgene- and nontransgene-expressing zebrafish embryos were evaluated. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 3 for corresponding morphology pictures. $p \leq 0.01$ (**); $p \leq 0.001$ (***)

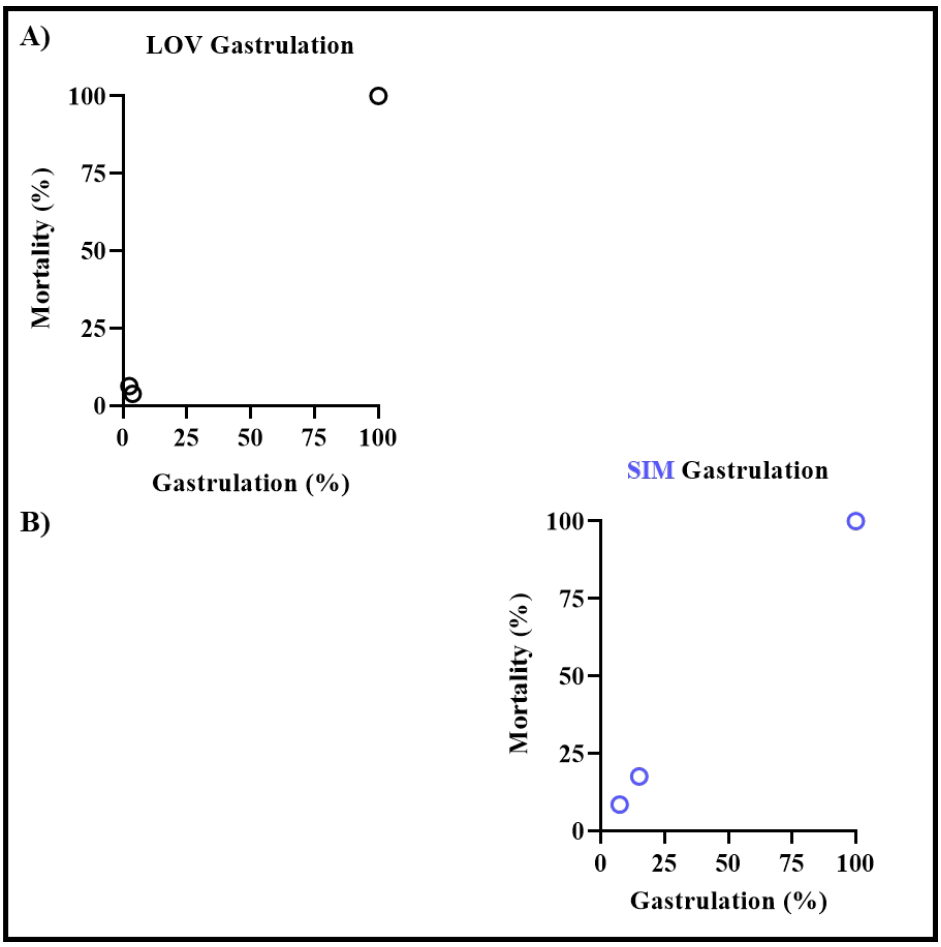

Figure 4-2. Association in the LOV and SIM Studies: Developmental Toxicity. Association with the incidences of cumulative mortality and developmental delay (gastrulation defects) from the (A) LOV study and (B) SIM study are displayed. Results are shown in different colors to distinguish the data by drug.

All zebrafish embryos in the nominal 1 µM (Very High) exposure groups of both the LOV and SIM studies experienced gastrulation defects on Day 1. No more than 15% of the unexposed embryos or embryos exposed to the drug-specific ERC Low treatment experienced gastrulation defects in the LOV or SIM studies (Figure 4-3). The incidence of embryos with gastrulation defects was significantly increased in the nominal 1 µM (Very High) (100%) exposure group of LOV compared to the Control exposure group (3%) and in the SIM-exposed embryos in the nominal 1 µM (Very High) (100%) exposure group compared to both the remaining exposure groups (Control: 15%, ERC Low: 7%).

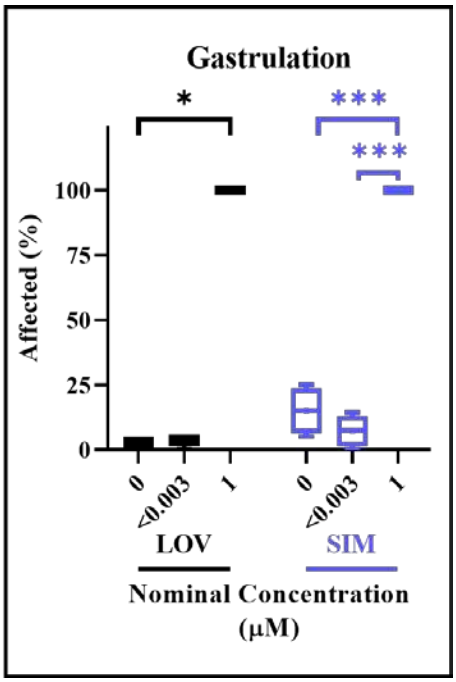

Figure 4-3. Developmental Progress in Individual Studies. Percent of embryos with developmental delay from the individual drug studies is displayed. Progress was evaluated based on presence of gastrulation defects in transgene- and nontransgene-expressing ZF embryos. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 3 for corresponding morphology pictures. $p \leq 0.05$ (*); $p \leq 0.001$ (***)

The proportion of embryos in the nominal 1 µM (Very High) exposure group that dechorionated early (40%) was significantly higher compared to the incidence in the Control group (1%) when exposed to PIT (Figure 4-4). Five percent of the embryos exposed to the nominal 2.4 x 10^{-4} µM (PIT ERC Low) treatment dechorionated early.

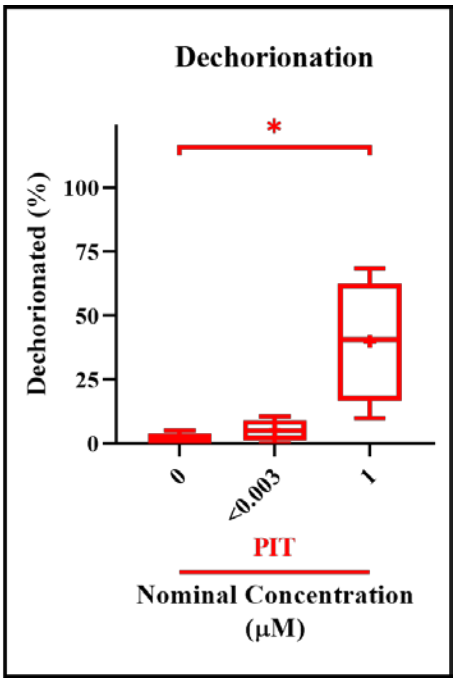

Figure 4-4. Dechorionation in Individual Studies. Percent of transgene- and nontransgene-expressing zebrafish embryos that dechorionated on Day 2 of the individual drug studies prior to manual dechorionation is displayed. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. $p \leq 0.05$ (*)

Muscle Abnormalities

The second category of observations assessed was myotoxicity, which included measuring the maximum velocity achieved in response to a touch stimulus and observing muscle fiber arrangements using birefringence. Dechorionated, nontransgene-expressing zebrafish embryos were gently prodded along their posterior axis on Day 2. Birefringence patterns were observed in transgene-expressing embryos on Day 3 using a polarized lens. PIT-exposed and FLUV-exposed embryos experienced both abnormalities and FENO-exposed embryos experienced one abnormality. Embryonic exposures to GEM, ATO, LOV, PRAV, ROS, and SIM did not exhibit any muscle abnormalities (Appendix 3). Representative morphology corresponding to significant findings from the FENO, FLUV, and PIT studies for this category are shown in Appendix 3.

The touch response (maximum achieved velocity) by embryos exposed to FLUV demonstrated a dose-dependent decrease as the nominal concentrations increased (Figure 4-5). The speed significantly decreased between the embryos in the Control group and the embryos exposed to the nominal 1 µM (Very High) treatment (from an average of 3.9 cm/s to 0.47 cm/s, respectively). There was no significant difference between the speeds observed from embryos in the Control groups compared to those of the embryos exposed at the nominal 2.4 x 10^{-4} µM (FLUV ERC Low) (2.5 cm/s). When measured with PIT treatment in zebrafish embryos, the response was not significantly different between the unexposed embryos (mean 3.9 cm/s) and the embryos exposed to the nominal 2.4 x 10^{-4} µM (PIT ERC Low) (5.2 cm/s) treatment. The velocity observed in the nominal 1 µM (Very High) exposure group was significantly different (0.92 cm/s) compared to the responses of embryos exposed to either the nominal 2.4 x 10^{-4} µM (PIT ERC Low) or Control exposure groups. Velocity was negatively associated with the incidence of early dechorionation in the PIT study and was negatively associated with the incidence in the FLUV study (Figure 4-6).

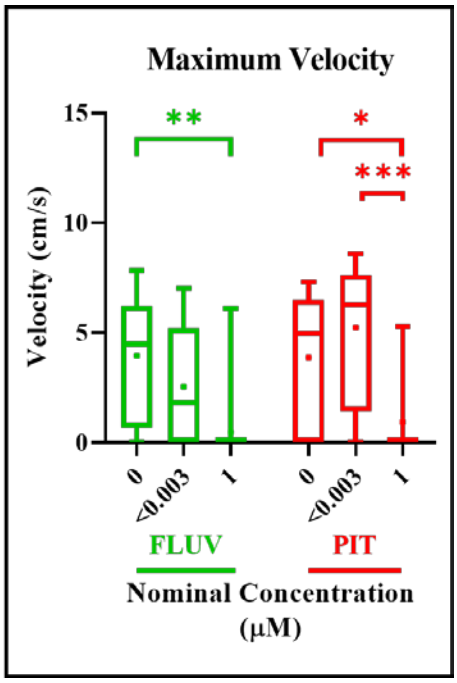

Figure 4-5. Maximum Velocity in Individual Studies. Maximum velocity of embryos from the individual drug studies is displayed. Only nontransgene-expressing zebrafish embryos were observed. A minimum of four embryos were assessed. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***)

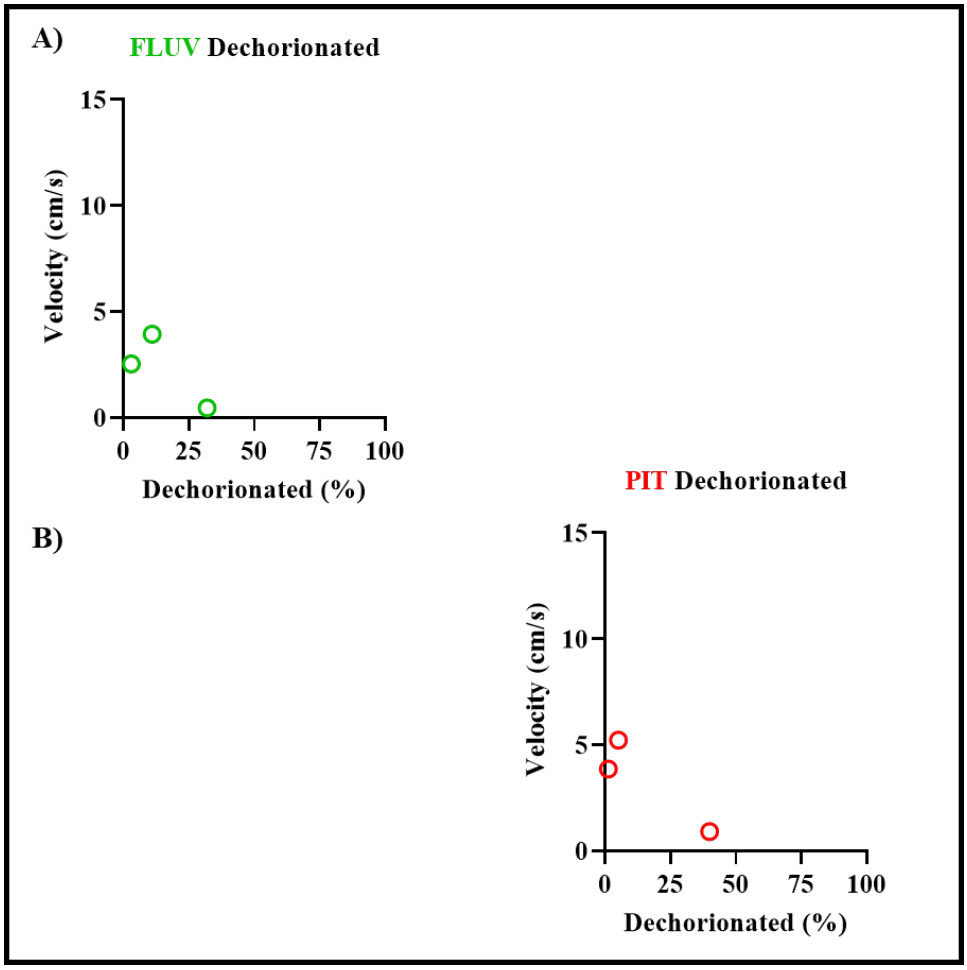

Figure 4-6. Association in the FLUV and PIT Studies: Muscle Abnormalities. Associations with the incidences of dechorionation and the maximum velocity from the (A) FLUV study and (B) PIT study are displayed. Results are shown in different colors to distinguish the data by drug.

Significant disruption of the muscle fibers, which caused loss of birefringence, was observed in embryos exposed to the nominal 1 µM (Very High) treatments of FENO, FLUV, or PIT (Figure 4-7). The frequencies of affected embryos in the FENO and PIT studies (11% and 15%, respectively) at the nominal 1 µM (Very High) exposure groups significantly increased compared to the ERC Low and Control exposure treatments of either drug study. The presence of muscle fiber mispatterning did not exceed 3% in the unexposed or in the ERC Low-exposed embryos of these three studies. The prevalence of embryos with birefringence abnormalities

exposed to FLUV was only significantly different between that of embryos exposed to the nominal 2.4 x 10^{-4} µM (FLUV ERC Low) and the nominal 1 µM (Very High) concentrations.

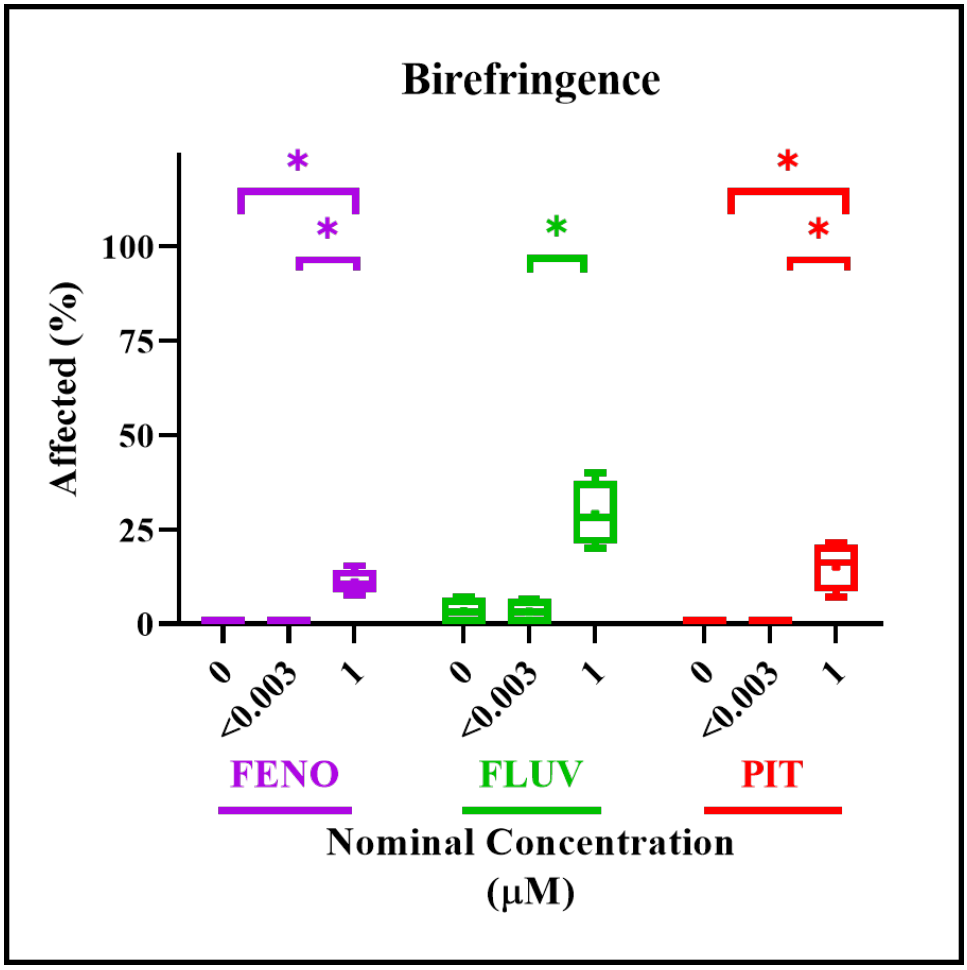

Figure 4-7. Abnormal Muscle Fiber Arrangement Using Birefringence in Individual Studies. Percent of embryos with abnormal muscle fiber patterns from the individual drug studies is displayed. Only transgene-expressing zebrafish embryos were evaluated. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 3 for corresponding morphology pictures. $p \leq 0.05$ (*)

Yolk Abnormalities

The third category of observations assessed was yolk abnormalities, which included yolk absorption and anterior to posterior (AP) axis determination. Both observations were made in dechorionated, transgene-expressing zebrafish embryos on Day 3, with each embryo positioned laterally. Three embryos were randomly selected from each replicate and their yolk areas were captured and measured. If any noticeable bend in the AP axis was visible, embryos were evaluated as affected. Significant defects of both kinds of abnormalities in this category were observed only in the embryos exposed to FLUV or PIT. Embryos exposed to FENO or SIM only experienced significant increases in their yolk areas; no axis deformities were observed. Embryonic exposures to GEM, ATO, LOV, PRAV, and ROS did not exhibit any yolk abnormalities (Appendix 3). Representative morphology corresponding to significant findings from the FENO, FLUV, PIT, and SIM studies for this category are shown in Appendix 3.

The amount of yolk remaining (measured as yolk area) was quantified and found to be significantly affected when zebrafish embryos were exposed to FENO, FLUV, PIT, or SIM (Figure 4-8). Like the pattern of birefringence abnormalities from the FLUV study, only the yolk areas of embryos exposed to FLUV at the nominal 2.4×10^{-4} µM (FLUV ERC Low) (2.4×10^5 µm^2) and the nominal 1 µM (Very High) (3.0×10^5 µm^2) exposure levels were significantly different from each other. The amount of yolk remaining in embryos from the nominal 2.4×10^{-5} (SIM ERC Low) (2.6×10^5 µm^2) significantly decreased compared to the yolk areas in the unexposed embryos (2.8×10^5 µm^2). The yolk areas significantly increased in FENO-exposed embryos at the nominal 1 µM (Very High) (2.9×10^5 µm^2) exposure level compared to embryos exposed in the nominal 2.8×10^{-3} µM (FENO ERC Low) (2.6×10^5 µm^2) and the Control groups

(2.6 x 10^5 µm^2). Yolk area was also significantly different between the nominal 1 µM (Very High) (3.0 x 10^5 µm^2) PIT treatment embryos and the respective Control group (2.6 x 10^5 µm^2).

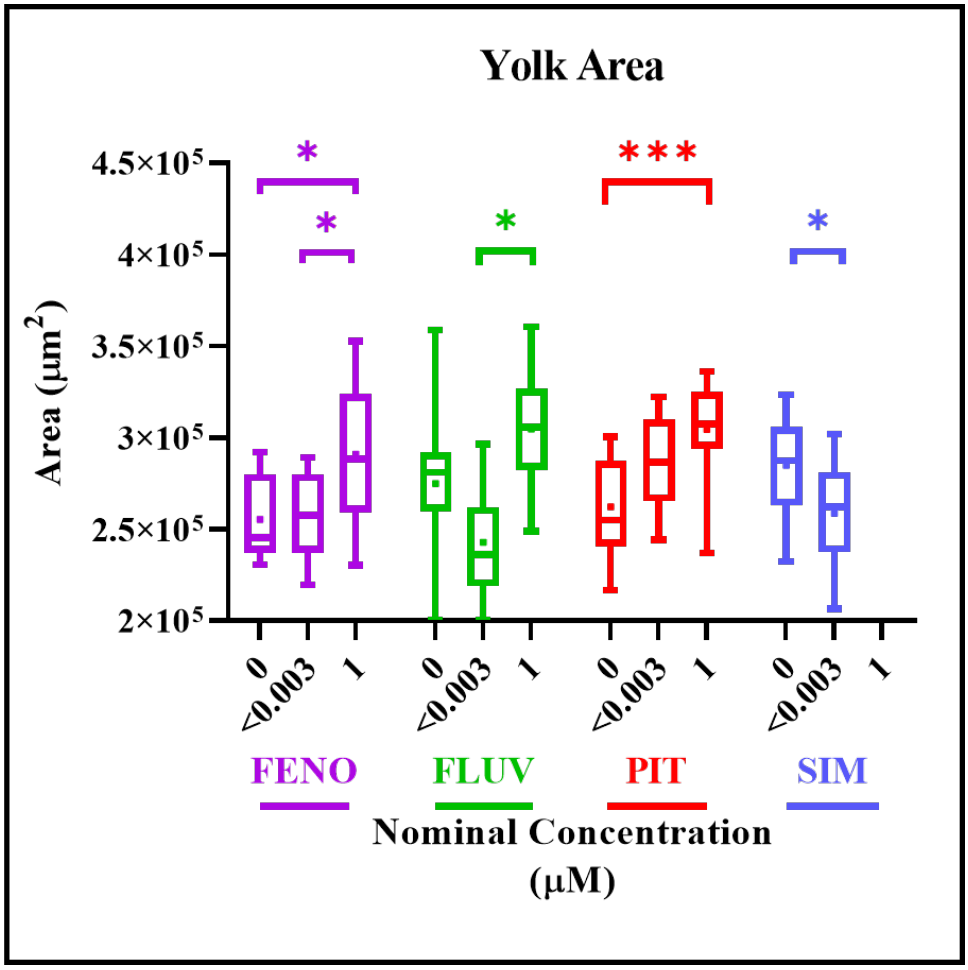

Figure 4-8. Abnormal Yolk Absorption in Individual Studies. Remaining yolk area of embryos from the individual drug studies is displayed. Only three transgene-expressing zebrafish embryo yolks were assessed. No data collected for this observation in the 1 µM SIM exposure group due to complete mortality. Results are displayed in different colors to distinguish the data by drug. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 3 for corresponding morphology pictures. $p \leq 0.05$ (*); $p \leq 0.001$ (***)

Significant AP abnormalities were observed in embryos exposed to the nominal 1 µM (Very High) exposure levels (Figure 4-9). Sixteen percent of embryos were significantly affected in this Very High level of PIT compared to embryos exposed to the nominal 2.4 x 10^{-4} µM (PIT ERC Low) and unexposed (Control) embryos. Sixty-six percent of the exposed embryos at the nominal 1 µM (Very High) level in the FLUV study exhibited abnormal AP axis deformities,

which was significantly greater compared to the incidences observed in either the nominal 2.4 x 10^{-4} µM (FLUV ERC Low) exposure or the Control groups. The prevalence of AP axis deformities did not exceed 2% in the nominal 2.4 x 10^{-4} µM (FLUV and PIT ERC Low) and unexposed treatments for either drug study.

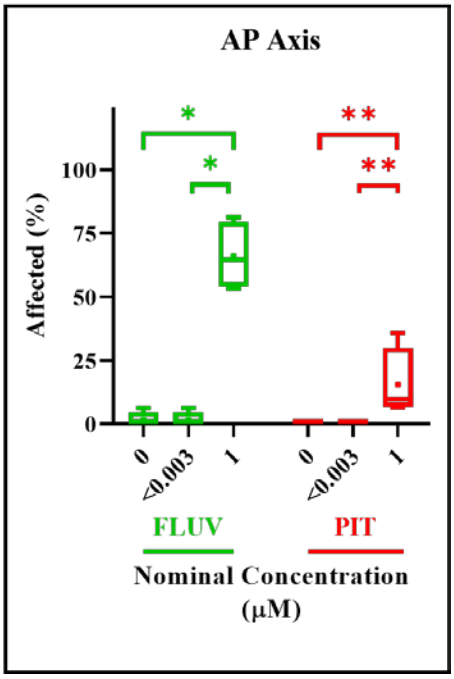

Figure 4-9. Altered Anterior-Posterior Axis Development in Individual Studies. Percent of embryos with abnormal angles to their anterior-posterior axis from the individual drug studies is displayed. Only transgene-expressing zebrafish embryos were evaluated. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 3 for corresponding morphology pictures. $p \leq 0.05$ (*), $p \leq 0.01$ (**)

Cardiovascular Abnormalities

The fourth category of observations assessed was cardiovascular abnormalities, which included vessel development and the presence of edema and hemorrhage. All were assessed in three-day old transgene-expressing zebrafish embryos. Intersegmental vessel (ISV) and subintestinal vein (SIV) development were assessed by observing the expression of green fluorescent protein. SIV development was subdivided into three categories: underdeveloped (SIV-under), overdeveloped (SIV-over), and missing (SIV-missing). The sum of the three

subcategories was statistically analyzed. The presence of pericardial or yolk edema or hemorrhage were evaluated as 'affected' or considered abnormal if visibly present under the bright field of a microscope. Embryos exposed to GEM, LOV, PRAV, ROS, and SIM did not exhibit any cardiovascular abnormalities (Appendix 3). Representative morphology corresponding to significant findings from the FENO, ATO, FLUV, and PIT studies for this category are shown in Appendix 3.

Only exposure to FLUV resulted in embryos exhibiting significant disruption of embryonic ISV development (Figure 4-10). The occurrence of these abnormalities significantly increased in embryos exposed to the nominal 1 µM (Very High) (11%) exposure level compared to the nominal 2.4 x 10^{-4} µM (FLUV ERC Low) (0%) exposure groups. Only three percent of unexposed embryos exhibited abnormal ISV development.

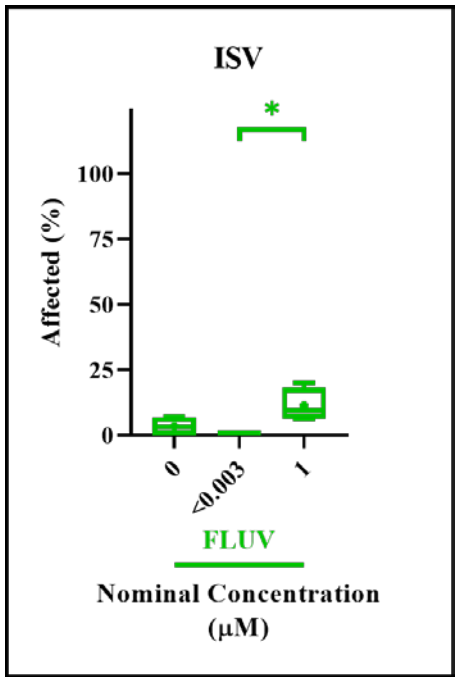

Figure 4-10. Abnormal Intersegmental Vessel Development in Individual Studies. Percent of transgene-expressing embryos with abnormal ISV development from the individual drug studies is displayed. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25^{th} - 75^{th} percentiles), and whiskers are minimum and maximum values. Refer to Appendix 3 for corresponding morphology pictures. $p \leq 0.05$ (*)

Missing SIV development was observed in embryos exposed to the nominal 1 µM (Very High) exposure concentrations of FENO, FLUV, and PIT (Figure 4-11). Like the pattern of abnormal ISV development, FLUV exposure resulted in a significant number of embryos missing SIVs when exposed to the nominal 1 µM (Very High) (16%) concentration compared to the nominal 2.4 x 10^{-4} µM (FLUV ERC Low) (0%) exposure level. Embryos missing SIVs when exposed to the nominal 1 µM (Very High) exposure concentrations of either FENO (18%) or PIT (13%) were significantly higher compared to the incidence of missing SIVs evaluated in embryos from the Control group and in ERC Low-exposed embryos. The occurrence of missing all SIVs in the Control group or in embryos exposed in the ERC Low levels in the FENO, FLUV, or PIT studies did not exceed 2%.

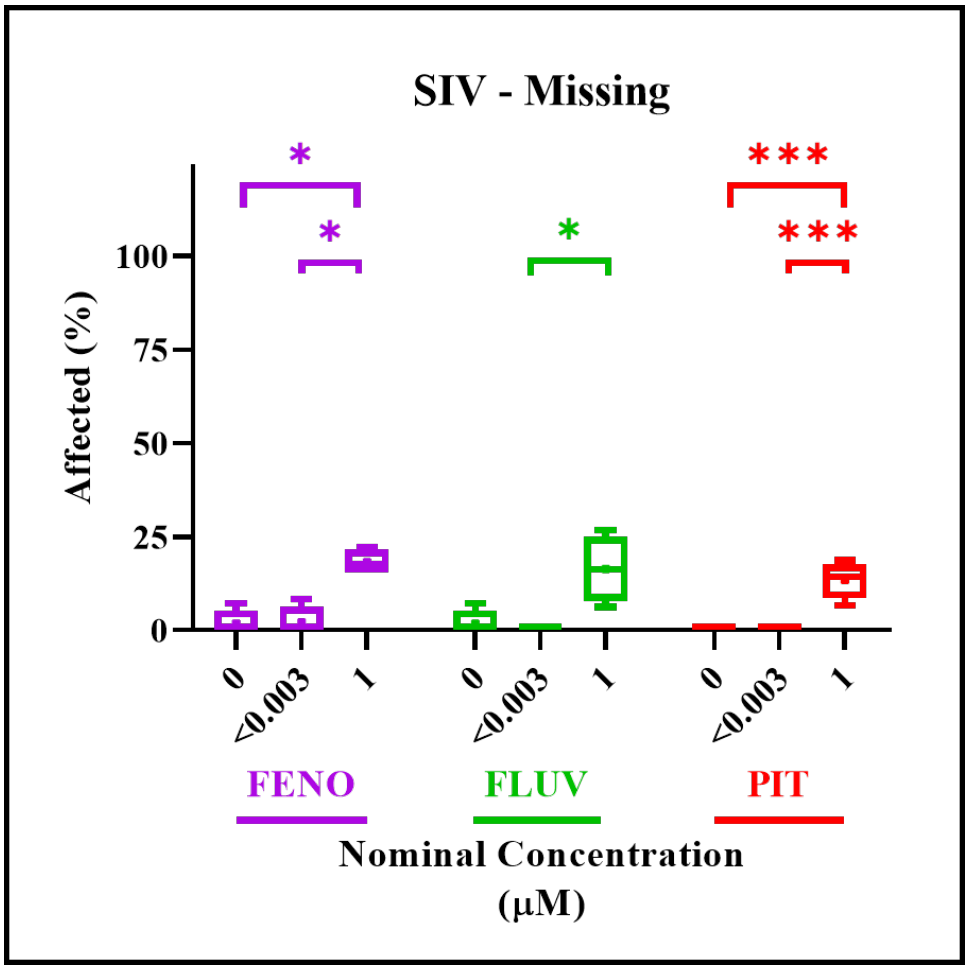

Figure 4-11. Abnormal Subintestinal Vein Development in Individual Studies: Missing. Percent of transgene-expressing embryos with missing SIVs from the individual drug studies is displayed. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 3 for corresponding morphology pictures. $p \leq 0.05$ (*); $p \leq 0.001$ (***)

The frequency of underdeveloped SIVs was significantly higher in embryos exposed in the nominal 1 µM (Very High) concentration (13%) in the FLUV study compared to the incidence in Control group embryos (3%) (Figure 4-12). The presence of overdeveloped SIVs was not significantly increased in any individual antilipidemic drug exposure (Appendix 3).

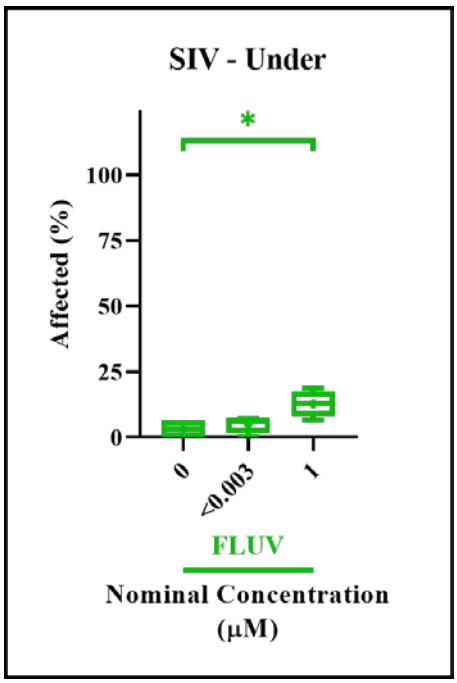

Figure 4-12. Abnormal Subintestinal Vein Development in Individual Studies: Underdeveloped Vessels. Percent of transgene-expressing embryos with underdeveloped SIVs from the individual drug studies is displayed. Affected embryos were evaluated if the SIV baskets were asymmetrical between the left and right sides of the body, exhibited less than five compartments, or if extension was less than 1/3 the length of the yolk. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. $p \leq 0.05$ (*)

After a summation of all subcategories of abnormal SIV development (under, over, and missing), embryos exposed to FENO and FLUV in the nominal 1 µM (Very High) groups experienced significantly more total SIV abnormalities (36% and 31%, respectively) compared to embryos in the ERC Low and Control groups (Figure 4-13). Total SIV abnormalities in embryos exposed in the nominal 2.8×10^{-3} µM (FENO ERC Low) concentration (19%) and unexposed embryos (16%) in the FENO study were not significantly different. Total SIV abnormalities in embryos exposed in the nominal 2.4×10^{-4} µM (FLUV ERC Low) concentration (5%) and unexposed embryos (6%) in the FLUV study were not significantly different.

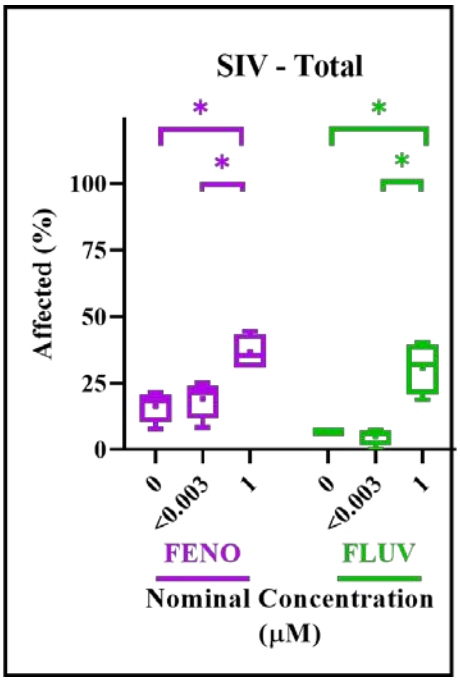

Figure 4-13. Abnormal Subintestinal Vein Development in Individual Studies: Total of Subcategories. Percent of transgene-expressing embryos with abnormally-developed SIVs from the individual drug studies is displayed. This total was calculated by summing the incidences from three subcategories: underdeveloped SIVs, overdeveloped SIVs, and missing SIVs. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25^{th} - 75^{th} percentiles), and whiskers are minimum and maximum values. $p \leq 0.05$ (*)

The presence of pericardial and yolk edema was significantly higher when embryos were exposed in the nominal 1 µM (Very High) treatment of FENO, FLUV, or PIT compared to the frequencies observed in the drug-specific ERC Low and Control exposure groups (Figures 4-14 and 4-15, respectively). No pericardial or yolk edema were observed in the unexposed treatment groups in these three studies. The presence of pericardial or yolk edema did not exceed 2% in the ERC Low exposure groups in any of the three studies. The frequency of pericardial edema was mostly higher compared to the frequency of yolk edema in the nominal 1 µM (Very High) groups in the three studies; FENO-, FLUV-, and PIT-exposed embryos exhibited pericardial edema at rates of 39%, 47%, and 22%, respectively; incidences of zebrafish embryos experiencing yolk edema was 26%, 19%, and 14%, respectively. In every instance where pericardial edema was observed, there was also yolk edema (Figures 4-16, 4-17, and 4-18).

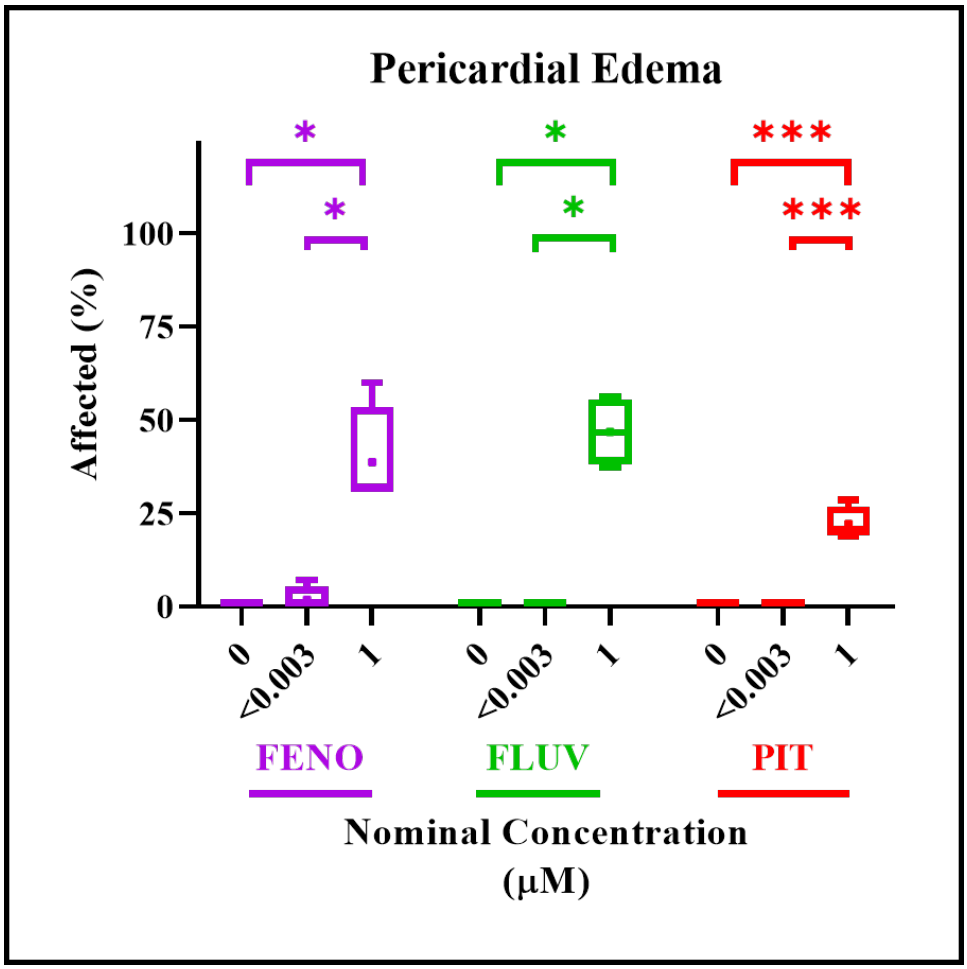

Figure 4-14. Presence of Pericardial Edema in Individual Studies. Percent of embryos with pericardial edema from the individual drug studies is displayed. Only transgene-expressing zebrafish embryos were evaluated. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 3 for corresponding morphology pictures. $p \leq 0.05$ (*); $p \leq 0.001$ (***)

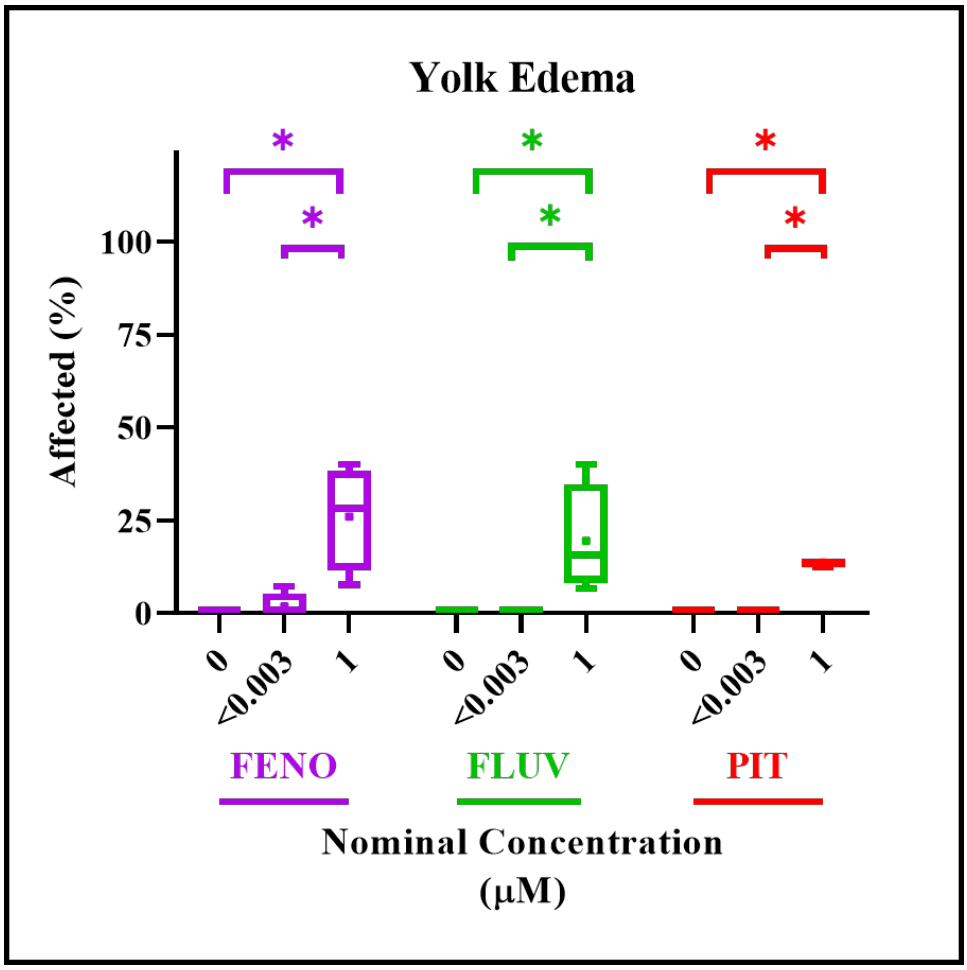

Figure 4-15. Presence of Yolk Edema in Individual Studies. Percent of transgene-expressing embryos with yolk edema from the individual drug studies is displayed. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25^{th} - 75^{th} percentiles), and whiskers are minimum and maximum values. Refer to Appendix 3 for corresponding morphology pictures. $p \leq 0.05$ (*)

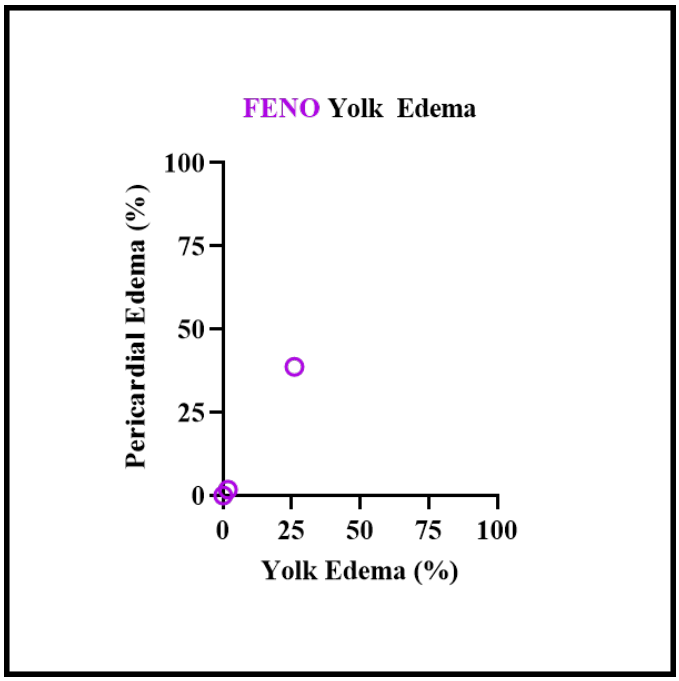

Figure 4-16. Association in the FENO Study: Cardiovascular Abnormalities. Association with the incidences of yolk edema and the presence of pericardial edema from the FENO mixture study is displayed. Results are shown in different colors to distinguish the data by drug.

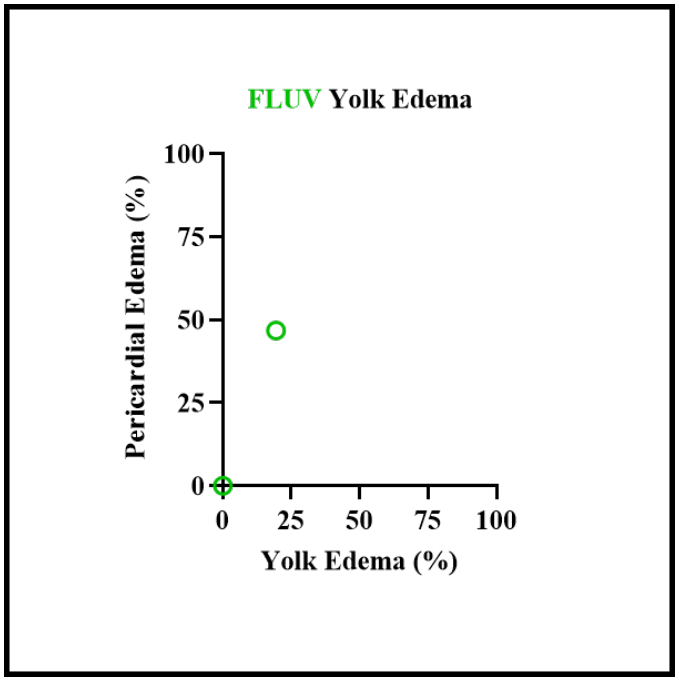

Figure 4-17. Association in the FLUV Study: Cardiovascular Abnormalities. Association with the incidences of yolk edema and the presence of pericardial edema from the FLUV mixture study is displayed. Results are shown in different colors to distinguish the data by drug.

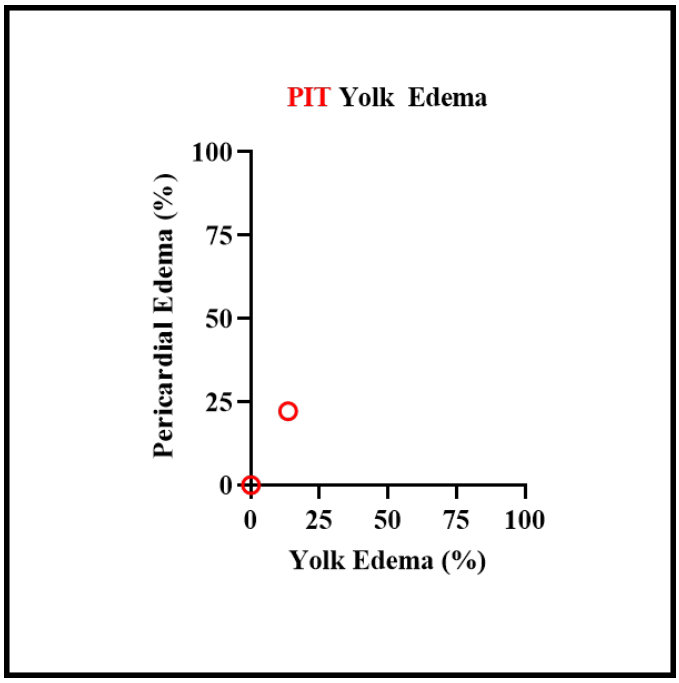

Figure 4-18. Association in the PIT Study: Cardiovascular Abnormalities. Association with the incidences of yolk edema and the presence of pericardial edema from the PIT mixture study is displayed. Results are shown in different colors to distinguish the data by drug.

The presence of hemorrhage was observed when embryos were exposed to individual exposures of ATO, FLUV, or PIT (Figure 4-19). This was the only significant effect observed in the ATO study. Embryos exposed in the nominal 1 µM (Very High) ATO treatment group exhibited hemorrhage in 34% of the population compared to less than 10% of embryos in the nominal 1.4 x 10^{-3} µM (ATO ERC Low) exposure and in the unexposed groups. The incidences of hemorrhage in embryos exposed in the nominal 1 µM (Very High) concentrations of either FLUV or PIT (67% or 72%, respectively) was significantly higher than those in the nominal 2.4 x 10^{-4} µM (FLUV and PIT ERC Low) and the unexposed Control groups. No more than 14% and 11% of embryos exhibited hemorrhage in the nominal 2.4 x 10^{-4} µM (FLUV and PIT ERC Low) and the unexposed Control groups, respectively.

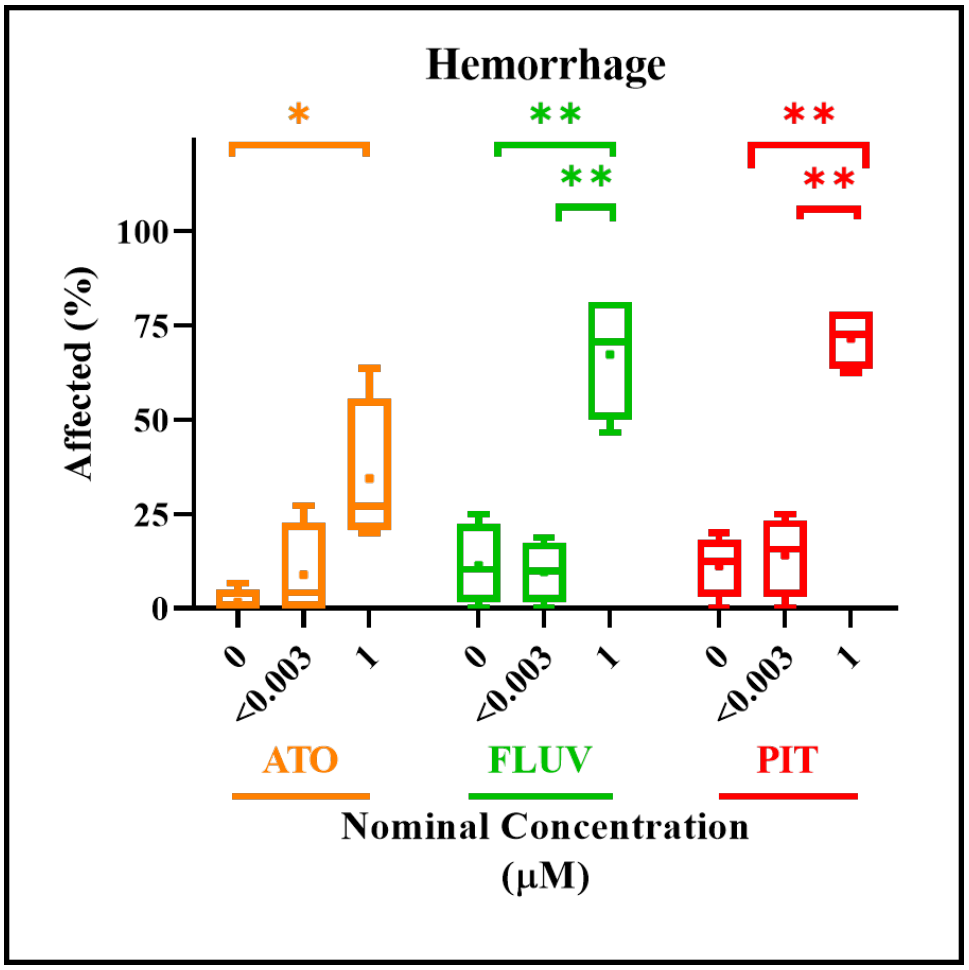

Figure 4-19. Presence of Hemorrhage in Individual ZF Studies. Percent of embryos with hemorrhage from the individual drug studies is displayed. Only transgene-expressing zebrafish embryos were evaluated. Results are displayed in different colors to distinguish the data by drug. Plots show the mean (+) and median (—) responses within each box, box edges define the interquartile range (25th - 75th percentiles), and whiskers are minimum and maximum values. Refer to Appendix 3 for corresponding morphology pictures. $p \leq 0.05$ (*); $p \leq 0.01$ (**)

Discussion

Effects from individual antilipidemic drug exposures were explored in zebrafish embryos to better understand the potencies of nine different antilipidemic drugs compared to the observed responses in mixture exposures. Although these studies focused on just zebrafish responses, future consideration should be given to replicating these studies with fathead minnows, based on their sensitive responses to exposure to the mixture in comparison to the ZF embryonic responses

Individual drug studies were conducted with ZF embryos because this model organism is gaining popularity in the field of toxicology (Lele & Krone, 1996; Vargas & Ponce-

Canchihuaman, 2017). This organism also contributes to the potential knowledge that can be gained across disciplines given the many benefits they offer to researchers including biochemists, geneticists, and cell biologists studying complex pathways or processes in fields including biochemistry, physiology, and behavior (Garcia, Noyes, & Tanguay, 2016). Online databases provide platforms for zebrafish researchers to compare findings (Garcia, Noyes, & Tanguay, 2016).

Exposures to GEM, PRAV, and ROS did not elicit any adverse lethal or sublethal effects. This GEM result was consistent with: (a) an EPA Ecological Structure Activity Response (ECOSAR) screening that concluded there was a low probability that GEM would exhibit an acute risk to fish (Sanderson, Johnson, Wilson, Brain, & Solomon, 2003); (b) a review of acute antilipidemic drug risks to the aquatic environment that concluded there was a low concern for GEM (and FENO) causing acute toxicity at ERCs (Brausch, Connors, Brooks, & Rand, 2012); and, with (c) a study assessing the impact of GEM on four aquatic systems that concluded even though GEM should be classified as harmful to aquatic organisms long term, it is not expected to be an acute concern to these systems (Zurita et al., 2007). Consideration should be given to longer GEM exposures in zebrafish embryos to include endocrine observations because previous exposures of GEM to adult goldfish at environmentally-relevant concentrations resulted in adverse endocrine effects from exposures as short as 96 hours or as long as 14 days (Mimeault et al., 2005). However, variation in species or age sensitivity may explain some observed differences because a 21-day exposure of GEM at an environmentally-relevant concentration to adult fathead minnows did not exhibit effects on lipid metabolism or sex steroids (Skolness et al., 2012).

Six of the nine drugs exhibited toxicity in ZF embryos, mainly at a relatively high concentration unrepresentative of those reported in environmental studies. Of the 15 observations assessed in each of these individual drug studies, significant adverse responses were observed in all four categories of abnormalities. These responses supported previous research concluding ZF embryos were permeable to low molecular weight compounds (Tobia, Gariano, De Sena, & Presta, 2013). LOV-exposed embryos only experienced one sublethal effect (gastrulation defect) and SIM-exposed embryos only experienced that same sublethal effect in addition to significant differences in yolk areas. The only significant observation in the ATO individual study was the presence of hemorrhage. Six sublethal criteria were significant in the FENO study, nine were significant in the PIT study, and 11 were significant in the FLUV study. However, in the FLUV study, some significant differences were not observed in comparison to those responses in the Control group but were observed compared to the lower exposure group.

Acute toxicity was only observed in embryos exposed to SIM or LOV. This also was associated with the incidence of significant embryonic gastrulation defects that were only recorded in the SIM and LOV studies. These two drugs are the only two that are currently prescribed to humans in their lactone forms (Kearney, Crawford, Mehta, & Radebaugh, 1993). Their pKa values are approximately three times higher than the remaining seven antilipidemic drugs prescribed in their hydroxy acid forms (Appendix 1). These two drugs were un-ionized at the pH of the exposure solutions and their greater lipophilicity likely facilitated their diffusion across cell membranes (Kearney et al., 1993; Yamasaki et al., 2009). This inactive prodrug lactone form converts to the active acid form once inside an organism (Kearney et al., 1993; Yamasaki et al., 2009).

Previous reports of acute toxicity values in zebrafish embryos exposed to any of these nine antilipidemic drugs was infrequent. Values for an LC_{50} of 0.84 µM (0.47 mg ATO/L) and an LC_{50} of 4.42 µM (1.59 mg FENO/L) were established in zebrafish embryos exposed to ATO or FENO during embryogenesis (1-4 dpf) (Chen et al., 2017). The only other vertebrate species used to assess lethal toxicity found in the research was a species of frog. *Xenopus laevis* larvae were used to determine LC_{50} values of 38.6 mg ATO/L and 52.2 mg LOV/L by Richards & Cole (2006). Other LC_{50} values were established in invertebrates: 1.5 mg ATO/L in *Hyalella azteca* (amphipod) (Dussault, Balakrishnan, Sverko, Solomon, & Sibley, 2008); 14.3 mg ATO/L in *Chironomus tentans* (insect) (Dussault et al., 2008); and, 1.18 mg SIM/L in *Palaemonetes pugio* (larval grass shrimp) (Key, Hoguet, Reed, Chung, & Fulton, 2008). Two orders of magnitude separated the minimum lethal concentration observed in zebrafish embryos from the LOV study of this project and less than one order of magnitude separated the minimum lethal concentration observed in the zebrafish embryos from the SIM study of this project compared to those of any of these previously published studies. It is not expected that individual exposures to any of the nine antilipidemic drugs tested here will cause acute lethality to fish at environmentally-relevant concentrations (Appendix 7).

Three individual drug studies yielded significant muscle system abnormalities: FENO, FLUV, and PIT. Both abnormal muscle fiber disarrangement and maximum achieved velocity were concurrently recorded only in PIT-exposed embryos at the Very High, non-environmentally relevant, concentration. Muscle fiber disarrangement in FENO-exposed embryos and differences in maximum velocity in FLUV-exposed embryos were detected. A previous FENO exposure (<2 dpf – 28 dpf) to FHM embryos did not result in any lethal or sublethal effects (Overturf et al., 2012). Muscle abnormalities in the SIM-, ATO-, LOV-, and PRAV-exposed embryos were not

observed, in contrast with previously published studies investigating statin myotoxicity *in vitro* or in human case studies (Bakar et al., 2018; Cao et al., 2009; Siriangkhawut, Tansakul, & Uchaipichat, 2017; Skottheim, Gedde-Dahl, Hejazifar, Hoel, & Asberg, 2008). However, *in vitro* exposure of human skeletal muscle cells to FLUV resulted in observed myotoxicity (Skottheim et al., 2008). One possible explanation for this difference, aside from *in vitro* versus *in vivo* models, was that the significant muscle defects observed in these studies involved exposure to drugs in their hydroxy acid form versus the previous study which assessed the lactone forms of several statins (Skottheim et al., 2008).

Optimization of a similar stimulus-response to touch assay in these studies, which measured startle responses and total distance moved in response to a vibration, was previously performed in zebrafish embryos to quickly assess an embryo's ability to escape predation following exposures to toxicants (Faria et al., 2019). This published study and the assay used here both revealed the ability of a ZF embryo to respond to a stimulus during development. The results reported here indicated responses at a more sensitive, younger age (48 hpf compared to 7-8 dpf) (Faria et al., 2019).

Zebrafish typically hatch between 48 and 72 hpf (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; Pina et al., 2018). Early dechorionation was only significantly increased in PIT-exposed embryos in the Very High exposure group. This observation may be a concern if embryos lose the protection of their chorion early because of exposure to toxicants in their environment but should be assessed separately in future work to rule out natural biological variability influencing the results.

Previous studies have highlighted the importance of yolk as storage for nutrients (lipids) and hormones in fish (Anderson, Carten, & Farber, 2011; Holtta-Vuori et al., 2010). When

absorption of yolk compounds is disrupted, organ system development, including the nervous system, may be affected as well as metabolism, growth, and survival (Holtta-Vuori et al., 2010; Raldua, Andre, & Babin, 2008). Zebrafish embryos previously exposed to 5 mg GEM/L (2 µM) exhibited an embryonic malabsorption syndrome that resulted in small-sized larvae with a large amount of yolk remaining. This was hypothesized to be related to impaired transport proteins in the yolk syncytial layer (Raldua et al., 2008). The hypothesis that exposure to an antilipidemic drug may cause yolk to not be fully absorbed was supported by the results reported here. These results of the previous zebrafish embryo study were consistent with the observed results from the GEM-exposed embryos in this project because embryos in this study were only exposed to a nominal concentration of 1 µM and no yolk abnormalities were observed. Surprisingly, the only significant observation in this project that occurred at a concentration close to those observed in the environment was the abnormal yolk absorption observed with SIM in embryos exposed to the nominal 2.4×10^{-5} µM (ERC Low SIM) concentration. Exposure of zebrafish embryos to PIT did significantly impact yolk absorption and because of the larger amount of yolk remaining, possibly induced a bend to the anterior-posterior axis at the nominal 1 µM concentration. Exposures to FENO, FLUV, and SIM resulted in either significant differences in the remaining yolk or abnormal angles to the AP axis, but not both.

Development of two important networks of vessels (SIVs and ISVs) was assessed because previous work suggested these networks were useful endpoints for screening drug toxicity (Serbedzija, Flynn, & Willett, 1999). Previous studies described angiogenesis of these two vessel systems in wildtype embryos (Childs, Chen, Garrity, & Fishman, 2002; Koenig et al., 2016) and the individual exposures of zebrafish embryos to FENO, FLUV, or PIT significantly disrupted this process in the studies reported here. Previous zebrafish embryo studies assessed

LOV, ROS, and SIM effects. Significantly disrupted ISV development was observed in embryos after 36-hour exposures to ROS at 10 µM, 10 times higher than the highest nominal exposure level in this project. However, zebrafish embryos exposed to LOV or SIM at 1 µM and 0.3 µM, respectively, exhibited disrupted ISV development at concentrations within the testing range of this project (Wang et al., 2010). Because significant differences in overdevelopment of SIVs were not observed here, the findings were not consistent with an *in vitro* statin study using human muscle and endothelial cells involving ATO, LOV, and SIM that identified angiogenic behavior at 1 µM and antiangiogenic behavior at higher tested concentrations (Frick et al., 2003). As a result of the project reported here, three antilipidemic drugs (FENO, FLUV, PIT) were identified in the zebrafish model that may be of concern due to their antiangiogenic potential. Additionally, subcategorizing abnormalities in SIV development is potentially more sensitive to adverse effects than assessing for all SIV abnormalities in aggregate, as demonstrated by the results of the PIT exposure study.

The presence of hemorrhage in ZF-exposed embryos was most commonly observed in the developing brain regions. Hemorrhage was not previously reported in zebrafish embryos exposed to FLUV or PIT. This presence in an ATO exposure reported here was consistent with previous research where zebrafish embryos experienced intracranial hemorrhage when exposed to 1 µM ATO (Gjini et al., 2011). In that same study, less than 5% of zebrafish embryos exposed to SIM at 5 x 10^{-3} µM exhibited hemorrhage (Gjini et al., 2011). Only 4% of zebrafish embryos exposed in the nominal 2.4 x 10^{-5} µM SIM (ERC Low) exhibited hemorrhage in this project. A previous zebrafish embryo study of ATO observed misshapen and dilated cerebral vessels in multiple brain regions with exposure to ATO (0.9 µM) (Eisa-Beygi, Hatch, Noble, Ekker, & Moon, 2013).

Exposures to 1 µM nominal concentrations of FENO, FLUV, and PIT resulted in similar patterns of edema in the zebrafish embryos. Pericardial and yolk edema were present more frequently at this concentration. These two abnormalities are common in zebrafish exposed to drugs (Hill, Bello, Prasch, Peterson, & Heideman, 2004; Reimers, Flockton, & Tanguay, 2004; Usenko, Harper, & Tanguay, 2007) and can be used as tools for early toxicity screening (Jeffries et al., 2015). These two types of edema were likely associated with heart dysfunction, but further investigation with more detailed cardiac observations is required.

In conclusion, toxic responses to nine individual antilipidemic drugs were assessed. Two drugs, FLUV and PIT, resulted in the most frequent number of sublethal effects in zebrafish embryos. Less attention may have been given to the environmental toxicity of FLUV because 90% of a human dose is excreted as metabolites (Center for Drug Evaluation and Research, 2000). However, the work reported here highlights the importance of conducting more research on this parent compound. PIT may have received less attention as an environmental contaminant because of its infrequent detection in the environment, but again, this work also highlights the potency of this drug. The zebrafish embryo exposures to FENO resulteFd in the next most frequent number of toxic responses in this project, but research pertaining to other fibrate drugs or to previously prescribed fibrate drugs were more frequent than FENO studies. The only response at a concentration approaching any that have been detected in the environment was observed in embryos where exposure to SIM resulted in significant decline in yolk absorption. An additional future project may also include evaluating nonadditive or potential synergistic responses when two or more of these drugs are present in the same exposure to better understand drug-drug interactions, including potential silencing interactions that may not be revealed by exposure to a larger number of drugs.

CHAPTER 5

CONCLUSIONS

Pharmaceuticals consumed by humans present an environmental concern because low, continually-present environmental concentrations of them (and their metabolites) may elicit impacts on nontarget organisms, including aquatic organisms, initially at unobservable effects. Long term adverse effects to aquatic life may result from these initial subtle changes that may be exacerbated when many pharmaceuticals are acting on an organism at once, which is environmentally the most common condition. These impacts may be misinterpreted as adaptation (Daughton & Ternes, 1999). Therefore, further investigation is warranted to assess any potential sublethal effects detected in aquatic organisms when exposed to pharmaceuticals. This project contributed to addressing this research gap by evaluating four categories of potential abnormalities that may occur during fish embryogenesis upon exposure to pharmaceuticals (Table 5.1). This research focused on two subclasses of the antilipidemic drug class and successfully assessed interspecies and inter-drug variability. Observations from mixture studies with all nine antilipidemic drugs were performed in embryos of two fish species, zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*); additionally, observations from individual drug exposures were performed with zebrafish embryos. Most significant criteria were observed in both the mixture studies and one or more of the single drug exposure studies. This suggests that single drug studies can elicit phenotypic responses that will also occur in a mixture study. However, few observations were significantly affected only by the drug mixture or only by a single drug exposure study -- but not both (i.e., disrupted ISVs or abnormal yolk absorption).This highlights the concern that responses from individual studies do not necessarily

indicate what would be observed in a mixture study. This also does not preclude the possibility that a mixture could have synergistic or cumulative ill effects.

Table 5.1

A Summary of Significant Observations from Each Study

Observation	ZF Mixture	FHM Mixture	Individual
Developmental Toxicity			
Cumulative Mortality	0.05 µM (Medium) 0.5 µM (High)	0.05 µM (Medium)	1 µM (Very High SIM)
Gastrulation Defect	0.5 µM (High)	NM	1 µM (Very High LOV, SIM)
Developmental Delay	NM	0.05 µM (Medium)	NM
Dechorionation	NS	NS	1 µM (Very High PIT)
Muscle Abnormalities			
Maximum Velocity	NS	NM	1 µM (Very High FLUV, PIT)
Muscle Fiber Mispatterning	0.005 µM (Low)	0.05 µM (Medium)	1 µM (Very High FENO, PIT)
Yolk Abnormalities			
Yolk Area	NS	NS	1 µM (Very High FENO, PIT) / 2.4×10^{-5} µM (ERC Low SIM)
Abnormal AP Axis Angle	0.005 µM (Low)	0.05 µM (Medium)	1 µM (Very High FLUV, PIT)
Cardiovascular Abnormalities			
ISV Development	0.005 µM (Low)	NM	NS
SIV-Missing Development	NS	NM	1 µM (Very High FENO, PIT)
SIV-Under Development	NS	NM	1 µM (Very High FLUV)
SIV-Over Development	NS	NM	NS
SIV-Total Development	NS	NM	1 µM (Very High FENO, FLUV)
Presence of Pericardial Edema	0.005 µM (Low)	0.05 µM (Medium)	1 µM (Very High FENO, FLUV, PIT)
Presence of Yolk Edema	0.005 µM (Low)	NS	1 µM (Very High FENO, FLUV, PIT)
Presence of Hemorrhage	0.005 µM (Low)	0.05 µM (Medium)	1 µM (Very High ATO, FLUV, PIT)
Heart Rate	NM	0.005 µM (Low) 0.05 µM (Medium)	NM

Note. Significant observations when compared to the unexposed (Control) group of embryos from each study is displayed for the four categories of abnormalities. For proportion data, four replicates per treatment group were statistically analyzed. Yolk area values from three embryos per replicate and maximum velocity values from a minimum of four embryos per replicate were statistically analyzed. All concentrations are nominal. NM = not measured, NS = nonsignificant, ISV = intersegmental vessels, SIV = subintestinal veins

According to these results, embryonic exposure to these two subclasses of drugs will likely not have an acute risk to fish at environmentally-relevant concentrations. Fathead minnow embryos experienced partial mortality when exposed to a mixture of antilipidemic drugs at the nominal 0.05 µM (Medium) group (51%). Complete mortality was observed in the embryos exposed in the nominal 0.05 µM (Medium) and 0.5 µM (High) concentrations of the zebrafish mixture study. Some of this observed acute toxicity may be attributed to exposures of LOV and SIM, because complete mortality was only observed in the zebrafish embryos exposed to higher tested concentrations [the nominal 1 µM (Very High) concentration]. No significant mortality was observed in other individual drug studies at any tested concentration. These toxic concentrations are orders of magnitude higher than concentrations detected in the environment.

Significant, sublethal criteria were observed in zebrafish embryos exposed to FENO, ATO, FLUV, LOV, PIT, and SIM. Most of these observations were noted in the mixture studies except for dechorionation, maximum velocity, yolk area, and development of the subintestinal veins (underdeveloped, missing, and total SIV only). Overdevelopment of SIVs was not observed at significant levels in any zebrafish embryo exposures in the mixture or individual studies. The development of the intersegmental vessels was only affected in embryos exposed in the zebrafish mixture study but not in any of the individual drug exposure studies. When fathead minnow embryos were exposed in the Ultra Low (0.0005 µM) nominal concentration of a mixture of nine antilipidemic drugs, the level closest to approaching concentrations observed previously in the environment, no significant effects were observed compared to those observed in unexposed embryos. Considering both the mixture and individual studies, only one observation, yolk absorption, was significantly different at a concentration lower than all the rest. This difference, when compared to the yolk absorption in unexposed embryos, was observed

only in zebrafish embryos exposed to the SIM ERC Low (nominal 2.4 x 10^{-5} µM) treatment; this level was the lowest exposure concentration of any drug in this project and was just 10 times the concentration of SIM previously recorded in effluent (Table 1.2 and Appendix 7).

Each of the observations within these four categories has the potential to result in abnormal fish development. The chorion provides a developing fish embryo with several benefits previously discussed and this perivitelline environment is where critical growth and development take place. If an embryo hatches too early or is too developmentally-delayed to naturally dechorionate, it may suffer adverse effects. Abnormal muscle development, yolk absorption, angiogenesis, or the presence of pericardial or yolk edema may each or may collectively inhibit an embryo's flight reaction which in turn would impact ability to escape predation. This may lead to population-wide effects as well.

These observed similarities and differences highlight the importance of continuing to assess individual agent and mixture toxicity. The results of this project support the conclusions of previous research performed with other pharmaceuticals (Horzmann, de Perre, Lee, Whelton, & Freeman, 2017; Schultz et al., 2011; Wolfe, Schorr, Hanson, Nelson, & Richards, 2015; Zenobio, Sanchez, Archuleta, & Sepulveda, 2014). Even though most of the effects observed here did not occur at environmentally-relevant exposure concentrations, additional research is encouraged to determine if more subtle damage is occurring at lower concentrations. The significant differences observed in the mixture exposures all occurred at concentrations lower than those responsible for the responses in the individual drug studies. Again, this comparison may be limited in scope due to the inconsistent concentrations evaluated in the individual and mixture studies.

Assessment of the fathead minnow abnormalities subjectively appeared more severe compared to those observed in similarly exposed zebrafish embryos. One possible explanation, specifically related to the increased severity of pericardial edema in fathead minnow embryos, may be related to cell differentiation and organ regeneration. Heart regeneration has been well studied previously in adult zebrafish and a few other teleost fish species, but has not been observed in the fathead minnow (Gonzalez-Rosa et al., 2017). Adult zebrafish heart cells have been shown to respecialize via a process that differs from that of other vertebrates that regenerate lost tissue, a process likely used earlier in their development (Sanchez-Iranzo et al., 2018). This process may be related to how the embryonic zebrafish heart patterns itself and how it compensates when normal heart development is disrupted (Sanchez-Iranzo et al., 2018). Therefore, while zebrafish embryos may be able to compensate when their developing heart is injured, because heart regeneration of adult fathead minnows has not been observed, fathead minnows may lack this ability to compensate for heart damage that occurs during embryonic development. Thus, fathead minnow embryos may exhibit a more severe pericardial edema phenotype as compared to that of zebrafish embryos. If abnormalities such as edema occur at lower concentrations in fish species that do not have this compensation ability, one could speculate this may impact the ability of other fish species to survive the transition to exogenous feeding, to successfully reach adulthood, to reproduce, and as well result in observed adverse effect in their offspring (if raised continuously with contaminants, meaning transgenerational effects). Certain abnormalities, such as pericardial edema, will have a greater impact on a fish reaching these life stages because, for example, a zebrafish embryo can only survive approximately five days post-fertilization without a functioning, healthy heart (Gonzalez-Rosa et al., 2017). Consideration should be given to other trophic levels as well; the invertebrate

cladoceran (*Daphnia magna*), was considered the most sensitive organism when exposed to antilipidemic drugs based on observed reproduction effects (Brausch, Connors, Brooks, & Rand, 2012).

Mixture studies are environmentally-relevant (Brain et al., 2004; Daughton, 2003; Emmanuel, Perrodin, Keck, Blanchard, & Vermande, 2005) and important to consider because humans prescribed statins often also take additional medications for other morbidities such as anticoagulants and antiplatelet medications (Eichel et al., 2010). This leads to a "poly-pharmacy" mixture in waste water effluent (Ramsey et al., 2014). Long-term biomonitoring and follow-up mixture studies with other drug classes or drugs within the antilipidemic class that have been previously removed from the market should be considered. For example, clofibric acid, a metabolite of the previously-prescribed clofibrate, is considered more toxic (acutely and chronically) to aquatic life than other antilipidemic drugs reviewed (Brausch et al., 2012). Fibrate and statin combination therapy in humans was previously investigated (Corsini, Bellosta, & Davidson, 2005).

This project supports the need to standardize embryonic testing parameters (Sfakianakis, Leris, Laggis, & Kentouri, 2011) and highlights the fact that there is no one test type that can assess toxicity of all contaminants (Jeffries et al., 2015). Study duration and starting embryonic age may greatly influence the outcome of a study. Standard testing methods for zebrafish (OECD, 1998; OECD, 2013) and for fathead minnows (EPA, 2002; OECD, 1998) include study durations of 4-10 days (zebrafish) or 7-9 days (fathead minnow) beginning with embryos as young as 30 minutes post fertilization up to less than 36 hours post fertilization. Use of a calculated benchmark concentration has been suggested (Hsieh et al., 2018) to streamline data analysis across labs and other disciplines. Standardized parameters for embryo-specific

observations, such as ones defined in whole effluent toxicity testing, would better characterize significant observations observed between labs as well. For example, previous work found that SIV development does not have standard patterning, especially between 2 and 4 dpf (Goi & Childs, 2016). Quantified criteria were described including vessel area coverage and number of compartments. Scoring in the project reported here adopted these concepts of defining coverage area and compartments to generate categories of SIV development but they were not identical, which may have resulted in variable sensitivities. This qualitative approach using subcategories may have limited application or generalizability because of this variability but this approach did use criteria that were still sensitive enough to detect significantly different responses between exposed and unexposed embryos. Angiogenesis of these two vessel families may still serve as a potentially useful endpoint for toxicological screening. An example would be ISV development, which is tightly controlled and predictably patterned and in which little plasticity exists (Goi & Childs, 2016). This development was not disrupted in any individual drug study in this project, but significant disruption was observed in the zebrafish mixture study at the nominal 0.005 µM (Low) concentration. Further investigation into the disrupted development of this essential vessel system that connects developing venous and arterial portions of the cardiovascular system is critical. Henn and Braunbeck (2011) also discussed the utility of another observation in fish embryo testing that was assessed in this project, early manual dechorionation.

In the study reported here, qualitative, dichotomous scoring of many observations was adopted in lieu of quantifiable observations as a result of logistical limitations. Likewise, the number of exposure concentrations was limited to keep the number of observations to a feasible level. Adequate assessment of developmental endpoints requires observation within a limited time window to ensure comparability of developmental stages. In order to assess a larger number

of observations for their potential utility in this study, the intensity of observations was necessarily limited. The use of barriers segregating sexes in spawning tanks that could be removed on the morning of test initiation may have reduced the variability in the starting age of the embryos. This increased control in the starting ages may also have limited the amount of embryo handling, which could adversely impact results if not standardized. Additional differences in dechorionation patterns (too early or too late) may be observed.

Despite these study limitations, this research was successful in identifying sensitive lethal and sublethal criteria in a short study duration that may direct future work. Some of the lack of test sensitivity resulting from the variability of measured responses may be attributed to interindividual responses to drugs like those observed in humans (Urquhart, Tirona, & Kim, 2007). To overcome this, embryonic testing can include more replicates and exposure concentrations, though both of these impose greater demands on logistic needs and requirements for qualified personnel. Consideration should be also given to testing metabolites and the other forms of these drugs (prodrug lactone or bioactive hydroxy acid forms). Perhaps these other compounds or forms also elicit adverse effects in aquatic organisms.

Statin use in humans is not expected to dramatically slow down (Kane, 2018), nor is the use of zebrafish as a model organism (Garcia, Noyes, & Tanguay, 2016; Han et al., 2018) or fish embryonic testing (Johns et al., 2009). A recent transition in the most popular statin prescribed in humans was observed in 2018; a shift from SIM to ATO was potentially due to the observed SIM-associated myotoxicity (Kane, 2018). At the time of this writing, six antilipidemic drugs were within the top 100 of ClinCalc DrugStat's Top 200 prescriptions of 2019 (ClinCalc, 2019). ATO ranked third behind thyroid and hypertension medicines; the same position it held in 2018 (ClinCalc, 2019). SIM ranked eighth, PRAV ranked 27th, ROS ranked 37th, and LOV ranked

72nd. The fibrates were also ranked: FENO was 67th and GEM was 132nd (ClinCalc, 2019). Notably missing in the ranks were FLUV and PIT.

Future work should begin with assessing interactions upon concomitant exposure of zebrafish and fathead minnow embryos to FLUV and PIT because they frequently exhibited significant adverse responses in the higher exposure groups and may contribute to the observed responses in mixture studies at lower levels. Gene expression studies including zebrafish RNA-sequencing could objectively support morphological effects observed *in vivo* and avoid subjective scoring differences (Pina et al., 2018; Zheng, Lu, & Zhao, 2018). These studies may detect subtle changes not observable morphologically within a short time frame but that can lead to significant effects during later development. Exploring cellular mechanisms related to the presence of hemorrhage or edema in the presence of these antilipidemic drugs and studying endothelial permeability may be an additional direction. Cholesterol regulates gap junctions within cell membranes and SIM and FLUV have been shown to interact with gap junctions in rat heart cells *in vitro* (Marsh et al., 2016; Zou et al., 2014). Research in atherosclerosis, a condition previously documented in salmon (Saunders et al., 1992), which may be present in other fish species, has more recently shifted from focusing solely on cholesterol build-up to the role of inflammatory processes (Lorenzatti & Retzlaff, 2016). Therefore, inflammatory pathway markers including inflammasomes or C-reactive protein may not only be identified in fish during exposures to antilipidemic drugs, but also at more sensitive levels (before morphological scoring is observed). Accuracy with the use of automatic screening software to assess zebrafish embryonic defects is improving and its use may reduce criticism related to researcher bias and allow for easy image storage for subsequent data analysis and reproducibility (Teixidó et al., 2018).

Summary

This research successfully assessed lethal and sublethal effects of nine individual antilipidemic drugs or a simultaneous mixture of all nine to embryos of two fish species, *Danio rerio* (zebrafish) and *Pimephales promelas* (fathead minnow). Several similar effects were observed in both fathead minnow and zebrafish embryos when they were exposed to mixtures of all nine drugs. Two lactone drugs, lovastatin (LOV) and simvastatin (SIM), resulted in both developmental delay (evaluated as gastrulation defects) and mortality in zebrafish embryos at a high exposure level. Two individual statin drugs, fluvastatin (FLUV) and pitavastatin (PIT), may be contributors to the observed toxicity in mixture studies. Zebrafish embryos exposed to the lowest evaluated concentration of simvastatin (200 times higher than recorded effluent concentrations) exhibited a significant impaired absorption of yolk. Seven significant observations of the 15 evaluated were present in the individual or mixture studies, but not in both types of exposures.

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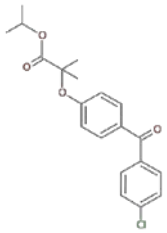
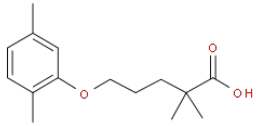
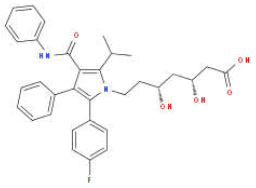
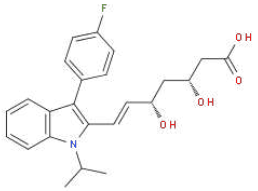
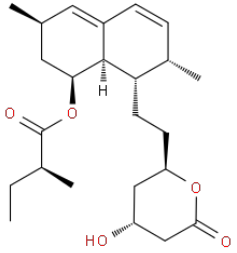
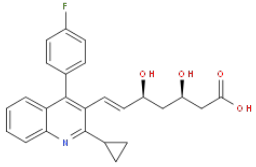
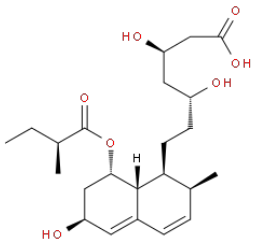
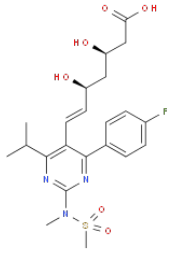
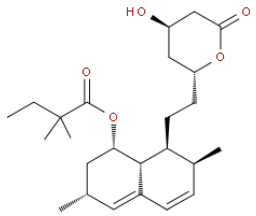
APPENDICES

APPENDIX 1

ANTILIPIDEMIC DRUG STRUCTURES

Table S-1

Molecular Structures for the Antilipidemic Drug Class

Fenofibrate	Gemfibrozil	Atorvastatin	Fluvastatin	Lovastatin
				
Pitavastatin	**Pravastatin**	**Rosuvastatin**	**Simvastatin**	
				

Note. Molecular structures redistributed here from ChEMBL in compliance with their Terms of Use (ChEMBL, 2019).

APPENDIX 2

NONSIGNIFICANT RESULTS AND CORRESPONDING PICTURES OF SIGNIFICANT MORPHOLOGY - MIXTURE STUDIES

Table S-2a

Nonsignificant Results from the ZF Mixture Study

Early Dechorionation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	23%	14%	5%	40%
Low	39%	26%	0%	58%
SIV - Under				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	10%	4%	6%	13%
Low	15%	9%	7%	25%
SIV - Missing				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	3%	0%	6%
Low	5%	6%	0%	13%
SIV - Over				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
Low	4%	4%	0%	7%
SIV - Total				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	11%	6%	6%	19%
Low	23%	11%	13%	38%
Maximum Velocity				
Group	Mean (cm/s)	Standard Deviation (cm/s)	Minimum (cm/s)	Maximum (cm/s)
Control	4.69	2.46	0.00	7.19
Low	3.49	2.87	0.00	6.75
Yolk Area				
Group	Mean (μm^2)	Standard Deviation (μm^2)	Minimum (μm^2)	Maximum (μm^2)
Control	2.65E+05	3.83E+04	2.07E+05	3.30E+05
Low	2.66E+05	5.86E+04	2.12E+05	4.09E+05

Note. Exposure to a mixture of nine antilipidemic drugs did not significantly impact these observations in ZF embryos.

Table S-2b

Nonsignificant Results from the FHM Mixture Study

Yolk Area				
Group	Mean (μm^2)	Standard Deviation (μm^2)	Minimum (μm^2)	Maximum (μm^2)
Control	4.36E+05	7.35E+04	2.97E+05	5.17E+05
Ultra Low	4.39E+05	7.83E+04	3.43E+05	5.90E+05
Low	4.31E+05	1.35E+05	2.10E+05	6.39E+05
Medium	4.83E+05	8.40E+04	3.22E+05	6.20E+05

Yolk Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
Ultra Low	0%	0%	0%	0%
Low	43%	23%	10%	61%
Medium	53%	45%	0%	100%

Note. Exposure to a mixture of nine antilipidemic drugs did not significantly impact these observations in FHM embryos.

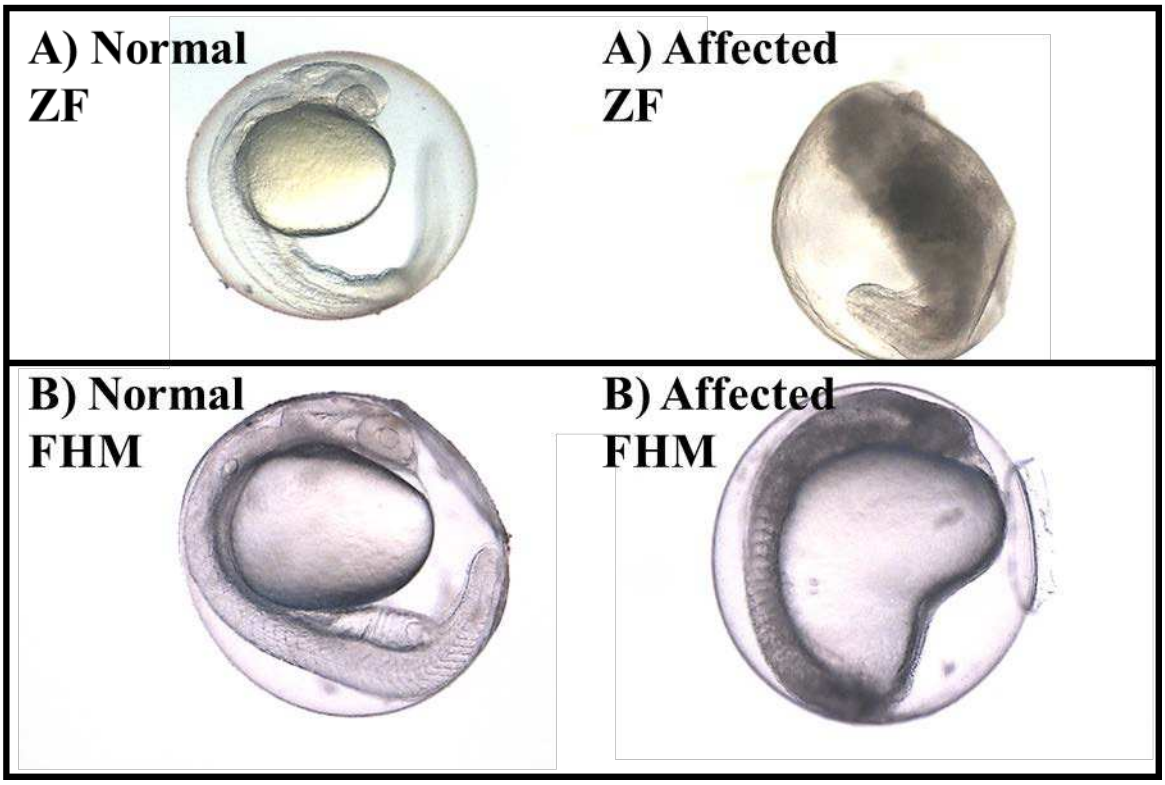


Figure S-2a. Corresponding Morphology from the ZF and FHM Mixture Studies: Cumulative Mortality. Pictures captured at 40x on Day 1 of either mixture study. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed in (A) ZF and (B) FHM embryos. The developing anterior portion of each embryo is oriented to the top of the chorion. This observation was statistically significant in both studies.

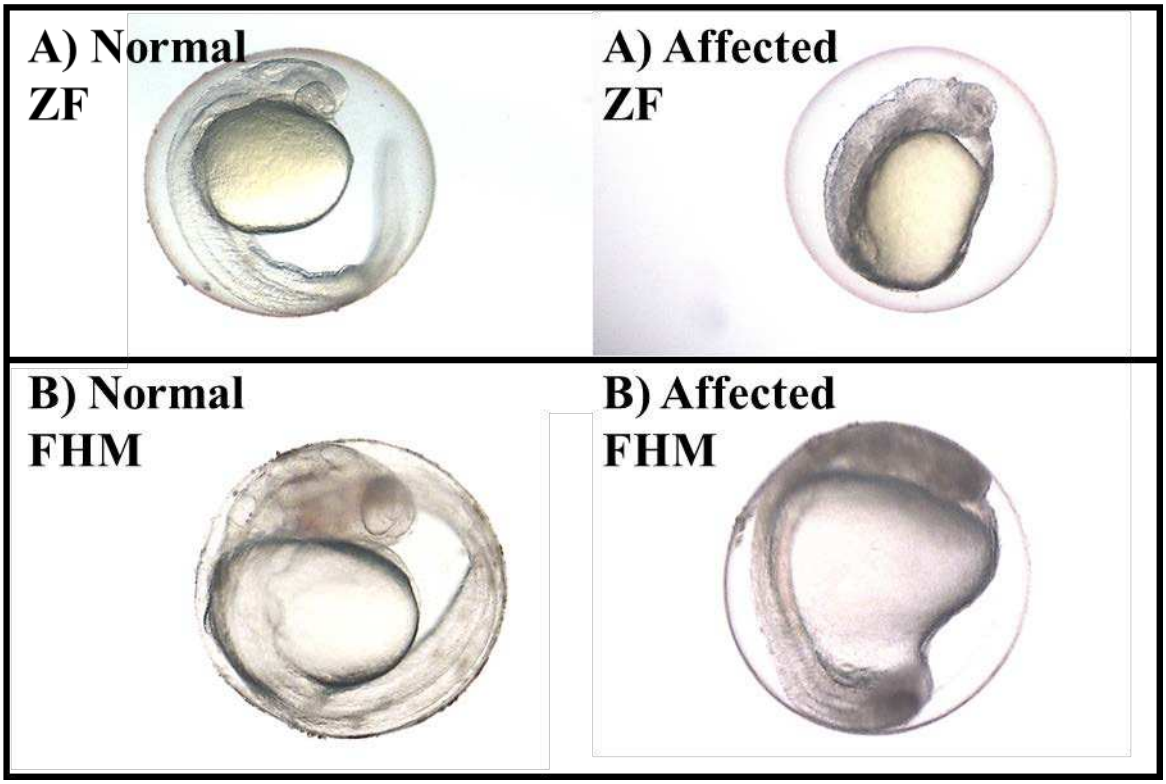


Figure S-2b. Corresponding Morphology from the ZF and FHM Mixture Studies: Developmental Progress. Pictures captured at 40x on Day 1 of the ZF mixture study and on Day 2 of the FHM mixture study. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed in (A) ZF and (B) FHM embryos. The developing anterior portion of each embryo is oriented to the top of the chorion. This observation was statistically significant in both studies.

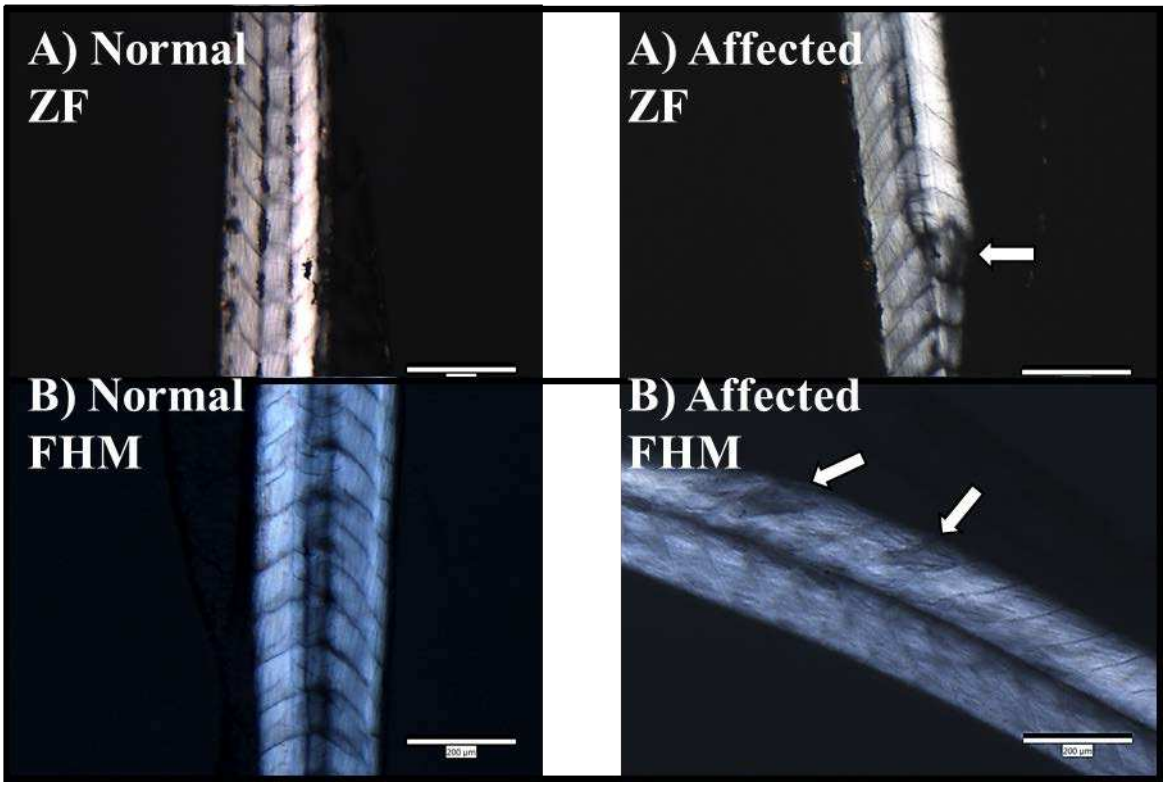


Figure S-2c. Corresponding Morphology from the ZF and FHM Mixture Studies: Muscle Fiber Arrangement Using Birefringence. Pictures captured at 90x on Day 3 of the ZF mixture study and on Day 6 of the FHM mixture study. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed in (A) ZF and (B) FHM embryos. A portion of the posterior end of each embryo is shown. This observation was statistically significant in both studies.

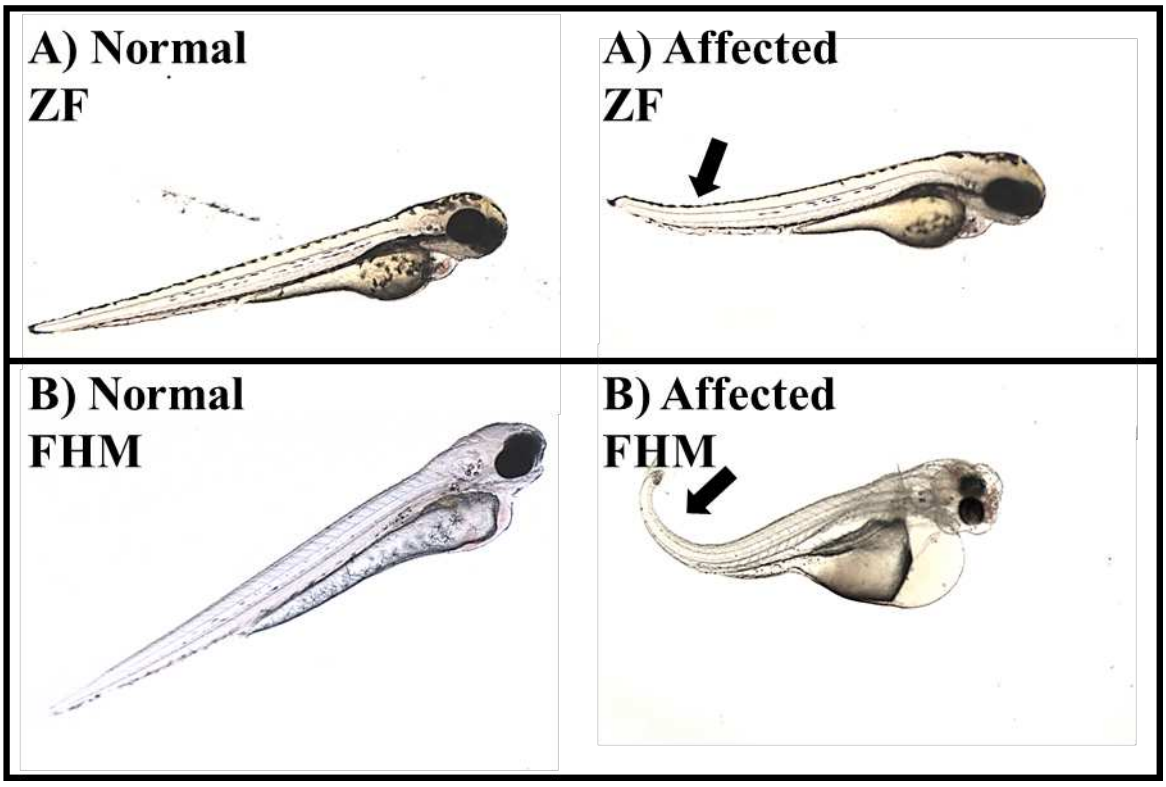


Figure S-2d. Corresponding Morphology from the ZF and FHM Mixture Studies: Altered AP Axis. Pictures captured at 25x on Day 3 of the ZF mixture study and at 20x on Day 6 of the FHM mixture study. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed in (A) ZF and (B) FHM embryos. The anterior region of each embryo is oriented to the right or top-right. This observation was statistically significant in both studies.

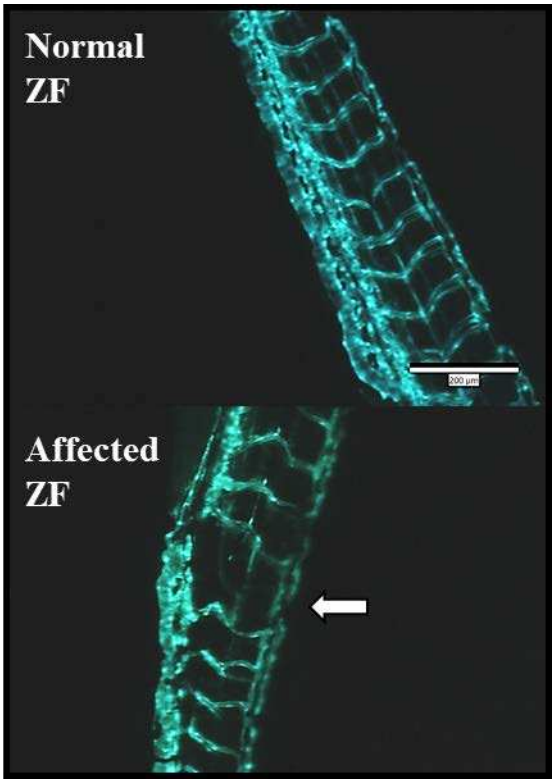


Figure S-2e. Corresponding Morphology from the ZF Mixture Study: Intersegmental Vessel Development. Pictures captured at 90x on Day 3 of the ZF mixture study. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. A portion of the posterior end of each embryo is shown. This observation was statistically significant only in the ZF mixture study.

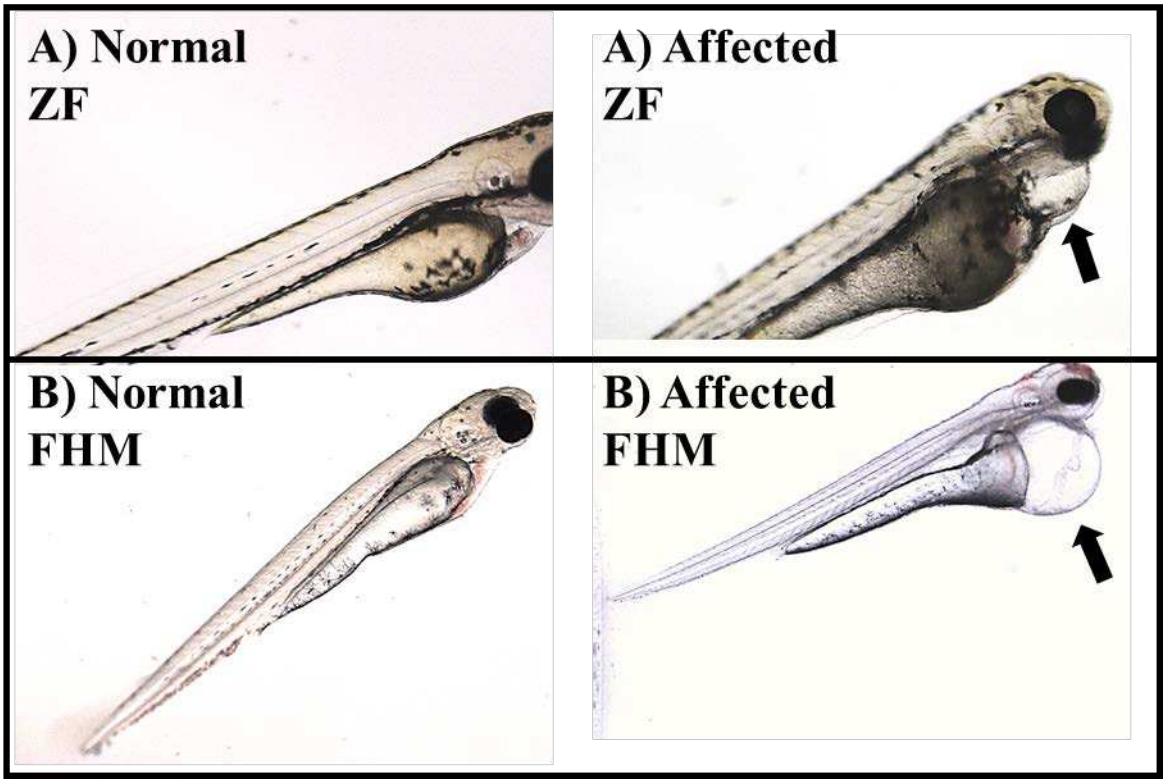


Figure S-2f. Corresponding Morphology from the ZF and FHM Mixture Studies: Presence of Pericardial Edema. Pictures captured at 40x on Day 3 of the ZF mixture study and at 20x on Day 6 of the FHM mixture study. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed in (A) ZF and (B) FHM embryos. The anterior region of each embryo is oriented to the top-right. This observation was statistically significant in both studies.

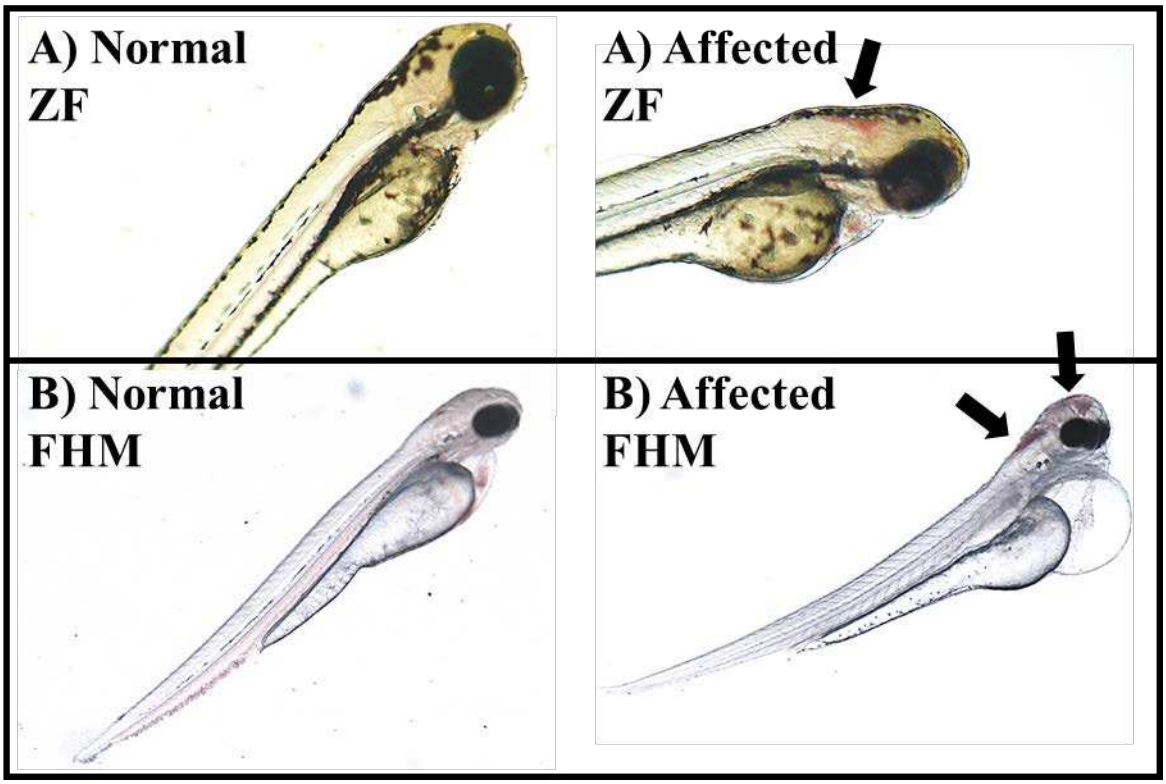


Figure S-2g. Corresponding Morphology from the ZF and FHM Mixture Studies: Presence of Hemorrhage. Pictures captured at 40x on Day 3 of the ZF mixture study and at 20x on Day 6 of the FHM mixture study. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed in (A) ZF and (B) FHM embryos. The anterior region of each embryo is oriented to the top-right. This observation was statistically significant in both studies.

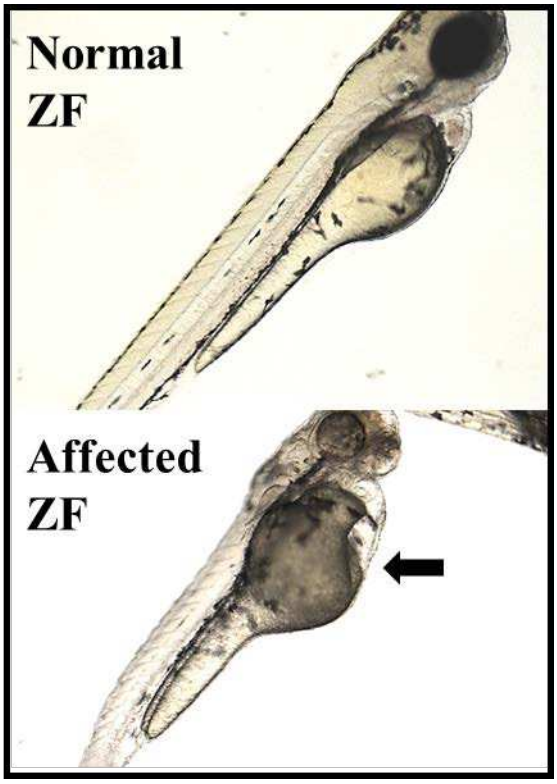


Figure S-2h. Corresponding Morphology from the ZF Mixture Study: Presence of Yolk Edema. Pictures captured at 40x on Day 3 of the ZF mixture study. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. The anterior region of each embryo is oriented to the top-right. This observation was statistically significant only in the ZF mixture study.

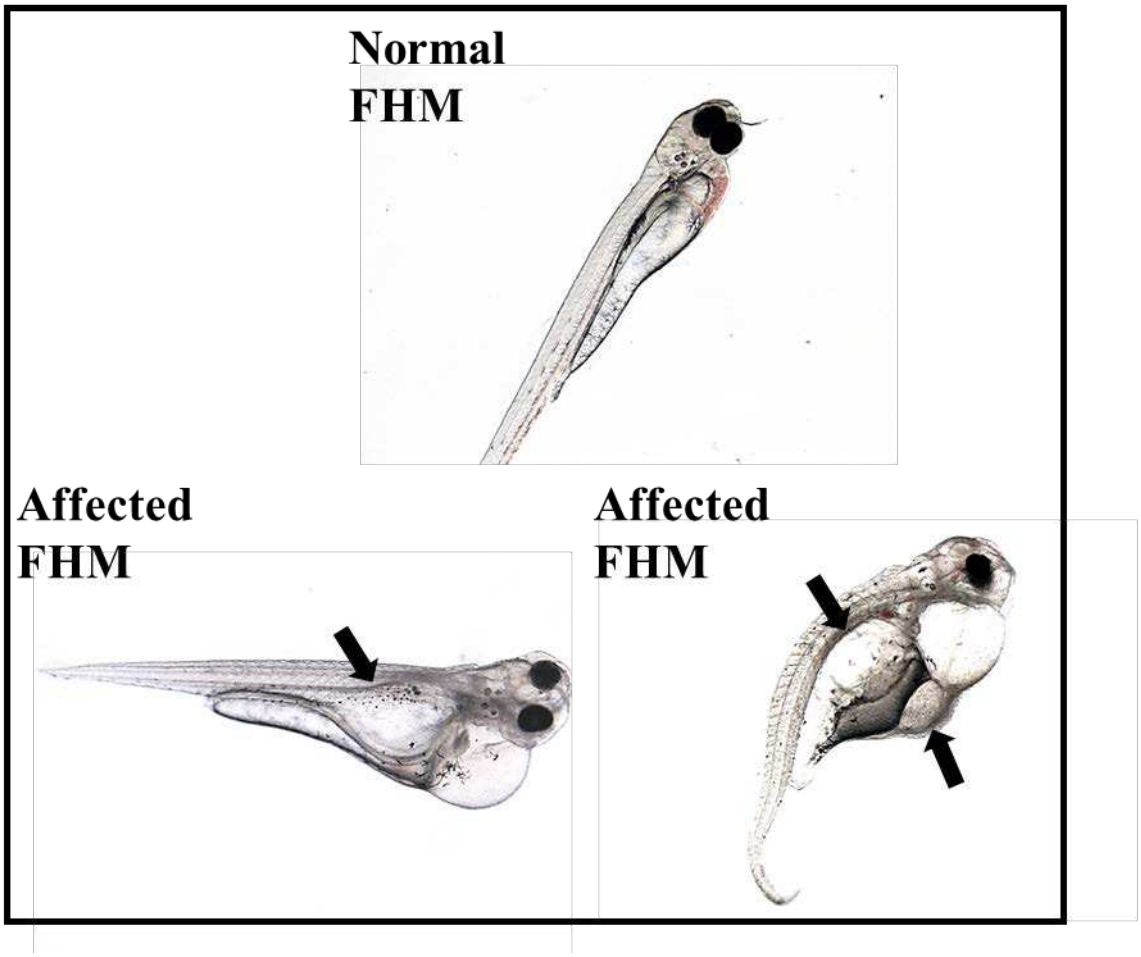


Figure S-2i. Corresponding Morphology from the FHM Mixture Study: Presence of Yolk Edema. Pictures captured at 20x on Day 6 of the FHM mixture study. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. The anterior region of each embryo is oriented to the top or right. This observation was statistically significant only in the FHM mixture study when one replicate was removed from the 0.05 µM (Medium) nominal exposure group. (See Chapter 3 for details.)

APPENDIX 3

NONSIGNIFICANT RESULTS AND CORRESPONDING PICTURES OF SIGNIFICANT MORPHOLOGY – INDIVIDUAL STUDIES

Table S-3a

Nonsignificant Results from the FENO Study

Hemorrhage				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	4%	0%	7%
ERC Low	0%	0%	0%	0%
Very High	8%	11%	0%	23%
Gastrulation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	13%	10%	0%	20%
ERC Low	6%	3%	5%	10%
Very High	14%	5%	10%	20%
Early Dechorionation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	8%	6%	0%	13%
ERC Low	15%	8%	6%	22%
Very High	12%	13%	0%	29%
AP Axis				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
Very High	2%	4%	0%	8%
Cumulative Mortality				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	14%	10%	0%	21%
ERC Low	13%	7%	5%	20%
Very High	26%	14%	11%	40%
ISV				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
Very High	0%	0%	0%	0%
SIV - Under				

Group	Mean	Standard Deviation	Minimum	Maximum
Control	13%	6%	7%	18%
ERC Low	15%	5%	8%	21%
Very High	16%	5%	10%	22%

SIV - Over

Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	4%	0%	7%
ERC Low	2%	4%	0%	7%
Very High	3%	5%	0%	10%

Maximum Velocity

Group	Mean (cm/s)	Standard Deviation (cm/s)	Minimum (cm/s)	Maximum (cm/s)
Control	4.38	3.14	0.00	7.78
ERC Low	4.21	4.12	0.00	14.7
Very High	4.37	2.44	0.00	7.19

Note. Exposure to FENO did not significantly impact these observations in ZF embryos.

Table S-3b

Nonsignificant Results from the GEM Study

Hemorrhage				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	5%	6%	0%	13%
ERC Low	7%	10%	0%	21%
Very High	5%	6%	0%	13%
Gastrulation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	3%	3%	0%	5%
ERC Low	4%	5%	0%	10%
Very High	3%	3%	0%	5%
Early Dechorionation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	37%	9%	26%	47%
ERC Low	35%	9%	25%	47%
Very High	45%	18%	21%	60%
AP Axis				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	3%	4%	0%	7%
Very High	5%	3%	0%	7%
Birefringence				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	2%	3%	0%	7%
Very High	0%	0%	0%	0%
Cumulative Mortality				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	4%	5%	0%	10%
ERC Low	10%	11%	0%	25%
Very High	4%	5%	0%	10%
ISV				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	2%	3%	0%	7%
Very High	2%	4%	0%	7%
Pericardial Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%

ERC Low	2%	3%	0%	7%
Very High	2%	3%	0%	6%
SIV - Missing				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	2%	3%	0%	7%
Very High	2%	4%	0%	7%
SIV - Over				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	3%	0%	6%
ERC Low	0%	0%	0%	0%
Very High	0%	0%	0%	0%
SIV - Under				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	12%	7%	6%	21%
ERC Low	10%	9%	0%	21%
Very High	12%	4%	6%	14%
SIV - Total				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	13%	8%	6%	21%
ERC Low	12%	9%	0%	21%
Very High	14%	6%	6%	21%
Yolk Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
Very High	0%	0%	0%	0%
Maximum Velocity				
Group	Mean (cm/s)	Standard Deviation (cm/s)	Minimum (cm/s)	Maximum (cm/s)
Control	4.27	2.69	0.00	6.84
ERC Low	5.11	2.67	0.00	8.03
Very High	3.58	3.17	0.00	7.40
Yolk Area				
Group	Mean (μm^2)	Standard Deviation (μm^2)	Minimum (μm^2)	Maximum (μm^2)
Control	2.67E+05	2.46E+04	2.31E+05	3.12E+05
ERC Low	2.49E+05	3.47E+04	2.10E+05	3.33E+05
Very High	2.55E+05	4.22E+04	1.76E+05	3.27E+05

Note. Exposure to GEM did not significantly impact these observations in ZF embryos.

Table S-3c

Nonsignificant Results from the ATO Study

Gastrulation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	4%	5%	0%	10%
ERC Low	11%	13%	0%	25%
Very High	6%	9%	0%	20%
Early Dechorionation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	6%	7%	0%	13%
Very High	0%	0%	0%	0%
AP Axis				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	15%	6%	7%	20%
ERC Low	4%	5%	0%	9%
Very High	6%	4%	0%	10%
Birefringence				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	9%	7%	0%	14%
ERC Low	7%	9%	0%	18%
Very High	11%	6%	6%	20%
Cumulative Mortality				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	4%	5%	0%	10%
ERC Low	11%	13%	0%	25%
Very High	8%	15%	0%	30%
ISV				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	2%	5%	0%	9%
Very High	0%	0%	0%	0%
Pericardial Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	3%	7%	0%	13%
ERC Low	2%	5%	0%	9%
Very High	0%	0%	0%	0%
SIV - Missing				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%

ERC Low	4%	5%	0%	9%
Very High	0%	0%	0%	0%
SIV - Over				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	4%	5%	0%	9%
ERC Low	4%	8%	0%	17%
Very High	0%	0%	0%	0%
SIV - Under				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	22%	16%	13%	45%
ERC Low	20%	3%	18%	25%
Very High	17%	12%	0%	27%
SIV - Total				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	26%	20%	13%	55%
ERC Low	28%	10%	19%	42%
Very High	17%	12%	0%	27%
Yolk Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	3%	0%	7%
ERC Low	4%	5%	0%	9%
Very High	0%	0%	0%	0%
Maximum Velocity				
Group	Mean (cm/s)	Standard Deviation (cm/s)	Minimum (cm/s)	Maximum (cm/s)
Control	4.78	2.63	0.00	8.76
ERC Low	3.16	3.31	0.00	7.70
Very High	3.81	3.54	0.00	9.55
Yolk Area				
Group	Mean (μm^2)	Standard Deviation (μm^2)	Minimum (μm^2)	Maximum (μm^2)
Control	2.88E+05	3.62E+04	2.28E+05	3.46E+05
ERC Low	2.69E+05	2.74E+04	2.11E+05	2.98E+05
Very High	2.85E+05	3.47E+04	2.35E+05	3.65E+05

Note. Exposure to ATO did not significantly impact these observations in ZF embryos.

Table S-3d

Nonsignificant Results from the FLUV Study

Gastrulation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	1%	3%	0%	5%
ERC Low	1%	3%	0%	5%
Very High	0%	0%	0%	0%
Early Dechorionation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	11%	19%	0%	39%
ERC Low	3%	3%	0%	6%
Very High	32%	28%	15%	74%
Cumulative Mortality				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	1%	3%	0%	5%
ERC Low	1%	3%	0%	5%
Very High	0%	0%	0%	0%
SIV - Over				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	3%	0%	6%
ERC Low	0%	0%	0%	0%
Very High	2%	3%	0%	6%

Note. Exposure to FLUV did not significantly impact these observations in ZF embryos.

Table S-3e

Nonsignificant Results from the LOV Study

Hemorrhage				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	4%	7%	0%	14%
ERC Low	8%	10%	0%	20%
Early Dechorionation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	17%	10%	5%	28%
ERC Low	25%	14%	5%	37%
AP Axis				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	3%	0%	7%
ERC Low	0%	0%	0%	0%
Birefringence				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	2%	3%	0%	7%
ISV				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
Pericardial Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	4%	0%	7%
ERC Low	5%	6%	0%	13%
SIV - Missing				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
SIV - Over				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	2%	3%	0%	7%
SIV - Under				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	19%	7%	13%	29%
ERC Low	17%	9%	7%	27%
SIV - Total				
Group	Mean	Standard Deviation	Minimum	Maximum

Control	19%	7%	13%	29%
ERC Low	18%	11%	7%	33%
Yolk Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
Maximum Velocity				
Group	Mean (cm/s)	Standard Deviation (cm/s)	Minimum (cm/s)	Maximum (cm/s)
Control	3.68	2.71	0.00	7.16
ERC Low	3.36	1.95	0.00	6.67
Yolk Area				
Group	Mean (μm^2)	Standard Deviation (μm^2)	Minimum (μm^2)	Maximum (μm^2)
Control	2.61E+05	2.29E+04	2.24E+05	3.00E+05
ERC Low	2.59E+05	2.61E+04	2.12E+05	3.18E+05

Note. Exposure to LOV did not significantly impact these observations in ZF embryos.

Table S-3f

Nonsignificant Results from the PIT Study

Gastrulation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	1%	3%	0%	5%
ERC Low	1%	3%	0%	5%
Very High	5%	4%	0%	10%
Cumulative Mortality				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	1%	3%	0%	5%
ERC Low	1%	3%	0%	5%
Very High	6%	5%	0%	10%
ISV				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
Very High	5%	6%	0%	13%
SIV - Under				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	8%	6%	0%	13%
ERC Low	9%	8%	0%	19%
Very High	13%	5%	7%	19%
SIV - Over				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	3%	0%	7%
ERC Low	0%	0%	0%	0%
Very High	0%	0%	0%	0%
SIV - Total				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	10%	9%	0%	20%
ERC Low	9%	8%	0%	19%
Very High	27%	8%	20%	38%

Note. Exposure to PIT did not significantly impact these observations in ZF embryos.

Table S-3g

Nonsignificant Results from the PRAV Study

Hemorrhage				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	3%	4%	0%	7%
ERC Low	6%	5%	0%	13%
Very High	0%	0%	0%	0%
Gastrulation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	1%	3%	0%	5%
ERC Low	3%	3%	0%	5%
Very High	1%	3%	0%	5%
Early Dechorionation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	4%	5%	0%	11%
ERC Low	4%	5%	0%	11%
Very High	0%	0%	0%	0%
AP Axis				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	2%	3%	0%	6%
Very High	1%	3%	0%	6%
Birefringence				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	3%	4%	0%	7%
Very High	1%	3%	0%	6%
Cumulative Mortality				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	1%	3%	0%	5%
ERC Low	3%	3%	0%	5%
Very High	1%	3%	0%	5%
ISV				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
Very High	0%	0%	0%	0%
Pericardial Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%

ERC Low	0%	0%	0%	0%
Very High	3%	4%	0%	7%
SIV - Missing				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
Very High	1%	3%	0%	6%
SIV - Over				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	3%	4%	0%	7%
Very High	0%	0%	0%	0%
SIV - Under				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	10%	6%	6%	19%
ERC Low	13%	5%	7%	20%
Very High	11%	11%	0%	25%
SIV - Total				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	10%	6%	6%	19%
ERC Low	16%	9%	7%	27%
Very High	13%	9%	6%	25%
Yolk Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	2%	3%	0%	7%
Very High	2%	3%	0%	7%
Maximum Velocity				
Group	Mean (cm/s)	Standard Deviation (cm/s)	Minimum (cm/s)	Maximum (cm/s)
Control	4.33	2.40	0.00	7.07
ERC Low	4.79	2.49	0.00	7.35
Very High	4.67	3.03	0.00	9.05
Yolk Area				
Group	Mean (μm^2)	Standard Deviation (μm^2)	Minimum (μm^2)	Maximum (μm^2)
Control	2.36E+05	3.14E+04	1.90E+05	2.99E+05
ERC Low	2.54E+05	4.94E+04	1.94E+05	3.46E+05
Very High	2.55E+05	2.11E+04	2.28E+05	3.03E+05

Note. Exposure to PRAV did not significantly impact these observations in ZF embryos.

Table S-3h

Nonsignificant Results from the ROS Study

Hemorrhage				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	4%	7%	0%	14%
ERC Low	5%	6%	0%	13%
Very High	9%	10%	0%	19%
Gastrulation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
Very High	3%	5%	0%	10%
Early Dechorionation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	3%	5%	0%	10%
ERC Low	8%	7%	0%	16%
Very High	8%	3%	5%	11%
AP Axis				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	3%	0%	6%
ERC Low	2%	4%	0%	7%
Very High	0%	0%	0%	0%
Birefringence				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	4%	0%	7%
ERC Low	3%	4%	0%	7%
Very High	4%	5%	0%	9%
Cumulative Mortality				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
Very High	3%	5%	0%	10%
ISV				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	2%	4%	0%	7%
Very High	0%	0%	0%	0%
Pericardial Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	3%	0%	6%

ERC Low	2%	4%	0%	7%
Very High	2%	4%	0%	8%
SIV - Missing				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	2%	4%	0%	7%
Very High	0%	0%	0%	0%
SIV - Over				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
Very High	0%	0%	0%	0%
SIV - Under				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	11%	11%	0%	25%
ERC Low	6%	9%	0%	19%
Very High	14%	10%	0%	23%
SIV - Total				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	11%	11%	0%	25%
ERC Low	8%	8%	0%	19%
Very High	14%	10%	0%	23%
Yolk Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
Very High	2%	4%	0%	8%
Maximum Velocity				
Group	Mean (cm/s)	Standard Deviation (cm/s)	Minimum (cm/s)	Maximum (cm/s)
Control	4.73	2.60	0.00	8.39
ERC Low	4.35	2.65	0.00	7.21
Very High	4.41	2.93	0.00	8.43
Yolk Area				
Group	Mean (μm^2)	Standard Deviation (μm^2)	Minimum (μm^2)	Maximum (μm^2)
Control	2.59E+05	3.12E+04	2.04E+05	3.22E+05
ERC Low	2.56E+05	2.13E+04	2.16E+05	2.88E+05
Very High	2.70E+05	3.40E+04	2.18E+05	3.39E+05

Table S-3i

Nonsignificant Results from the SIM Study

Hemorrhage				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	7%	9%	0%	18%
ERC Low	4%	7%	0%	14%
Early Dechorionation				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	12%	9%	0%	20%
ERC Low	7%	7%	0%	17%
AP Axis				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	4%	5%	0%	9%
ERC Low	0%	0%	0%	0%
Birefringence				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	6%	4%	0%	9%
ERC Low	4%	4%	0%	8%
ISV				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	2%	4%	0%	8%
ERC Low	0%	0%	0%	0%
Pericardial Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	6%	9%	0%	18%
ERC Low	5%	7%	0%	14%
SIV - Missing				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	4%	5%	0%	9%
ERC Low	5%	4%	0%	8%
SIV - Over				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	0%	0%	0%	0%
SIV - Under				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	11%	12%	0%	27%
ERC Low	7%	6%	0%	15%
SIV - Total				
Group	Mean	Standard Deviation	Minimum	Maximum

Control	15%	16%	0%	36%
ERC Low	13%	10%	0%	23%
Yolk Edema				
Group	Mean	Standard Deviation	Minimum	Maximum
Control	0%	0%	0%	0%
ERC Low	2%	4%	0%	7%
Maximum Velocity				
Group	Mean (cm/s)	Standard Deviation (cm/s)	Minimum (cm/s)	Maximum (cm/s)
Control	2.94	2.87	0.00	7.45
ERC Low	3.73	3.26	0.00	8.16

Note. Exposure to SIM did not significantly impact these observations in ZF embryos.

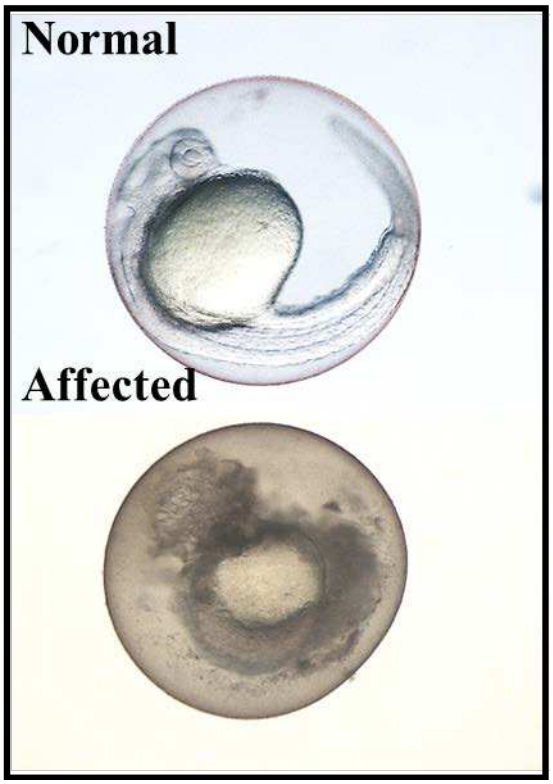


Figure S-3a. Corresponding Morphology from the Individual Studies: Cumulative Mortality. Pictures captured at 40x on Day 1 of the individual studies. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. The developing anterior portion of each embryo is oriented to the left or top-left of the chorion. This observation was statistically significant in the lovastatin (LOV) and simvastatin (SIM) drug studies.

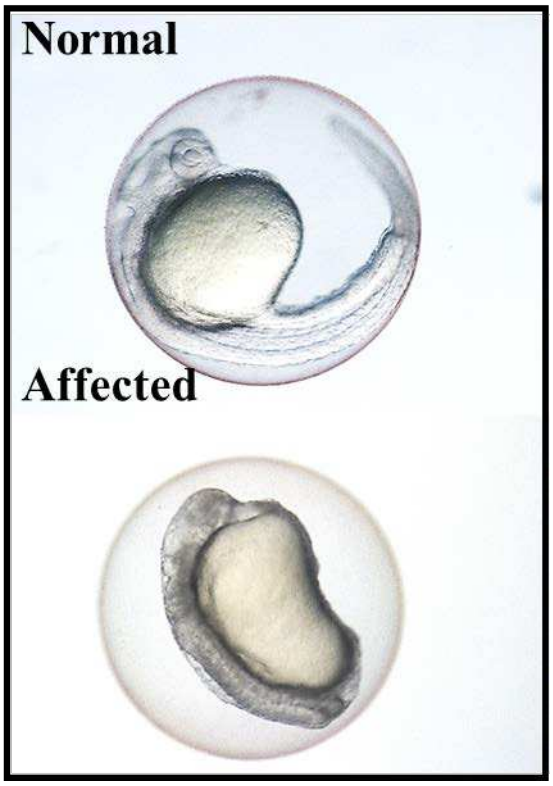


Figure S-3b. Corresponding Morphology from the Individual Studies: Developmental Progress. Pictures captured at 40x on Day 1 of the individual studies. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. The developing anterior portion of each embryo is oriented to the top of the chorion. This observation was statistically significant in the lovastatin (LOV) and simvastatin (SIM) drug studies.

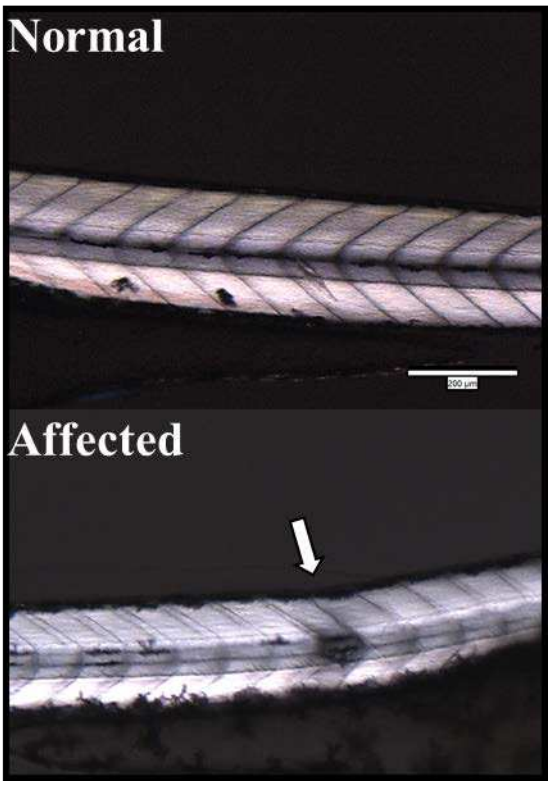


Figure S-3c. Corresponding Morphology from the Individual Studies: Muscle Fiber Arrangement Using Birefringence. Pictures captured at 90x (Normal) and 63x (Affected) on Day 3 of the individual studies. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. A portion of the posterior end of each embryo is shown. This observation was statistically significant in the fenofibrate (FENO), fluvastatin (FLUV), and pitavastatin (PIT) drug studies.

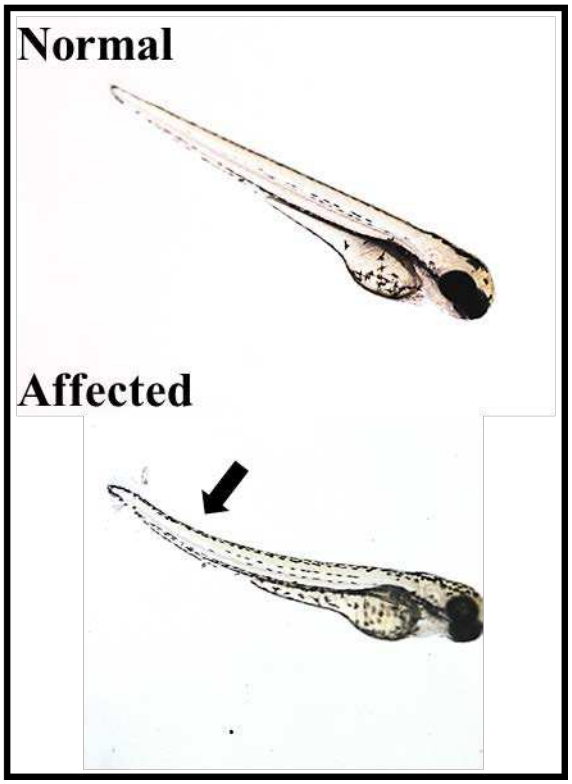


Figure S-3d. Corresponding Morphology from the Individual Studies: Altered AP Axis. Pictures captured at 20x on Day 3 of the individual studies. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. The anterior region of each embryo is oriented to the right. This observation was statistically significant in the fluvastatin (FLUV) and pitavastatin (PIT) drug studies.

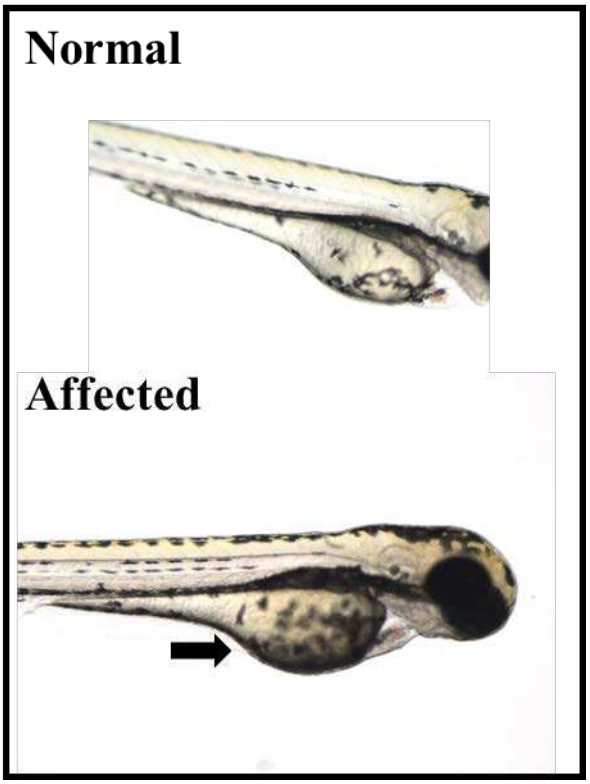


Figure S-3e. Corresponding Morphology from the Individual Studies: Yolk Areas. Pictures captured at 40x on Day 3 of the individual studies. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. The anterior region of each embryo is oriented to the right. The arrow indicates a large amount of yolk remaining at test termination, which was quantified by outlining the yolk (including the yolk extension) in ImageJ software. This observation was statistically significant in the fenofibrate (FENO), fluvastatin (FLUV), pitavastatin (PIT), and simvastatin (SIM) drug studies.

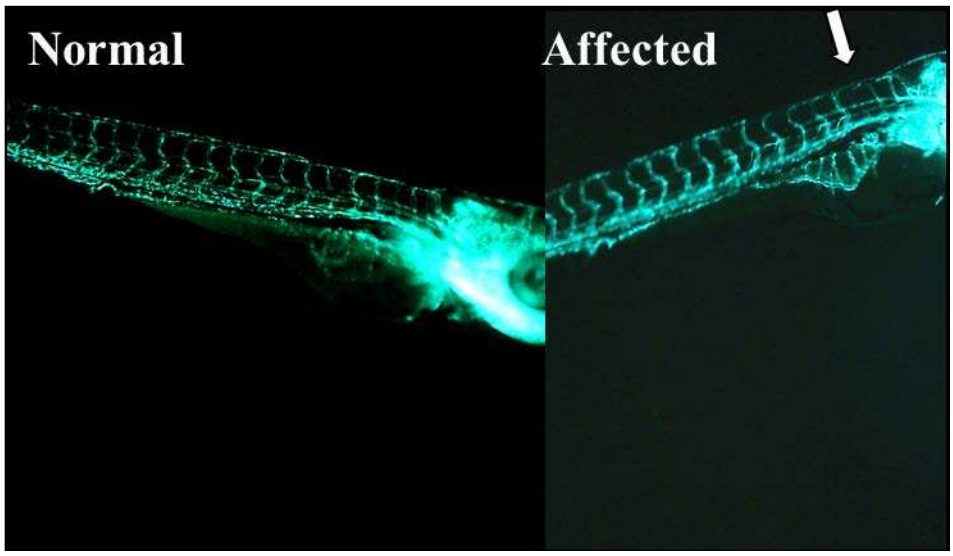


Figure S-3f. Corresponding Morphology from the Individual Studies: Intersegmental Vessel Development. Pictures captured at 40x on Day 3 of the individual studies. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. The anterior region of each embryo is oriented to the right. This observation was statistically significant in the fluvastatin (FLUV) drug study.

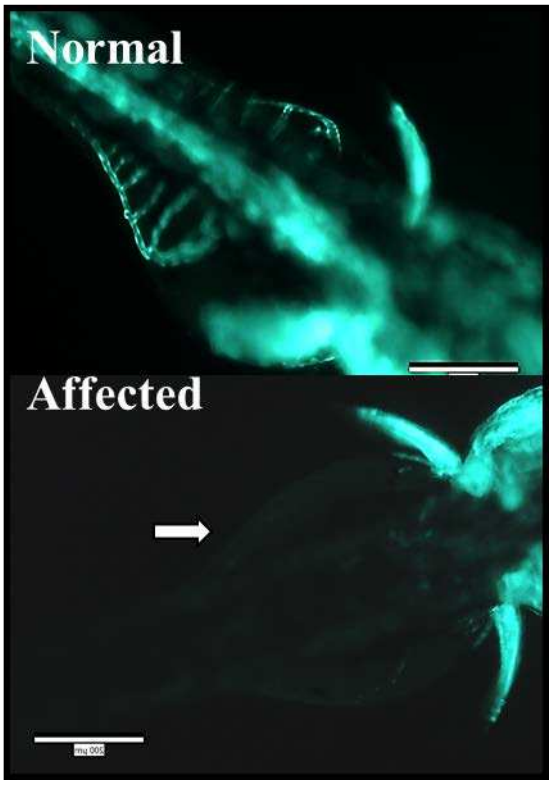


Figure S-3g. Corresponding Morphology from the Individual Studies: Subintestinal Vein Development: Missing. Pictures captured at 90x on Day 3 of the individual studies. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. The anterior region of each embryo is oriented to the bottom-right or top-right. This observation was statistically significant in the fenofibrate (FENO), fluvastatin (FLUV), and pitavastatin (PIT) drug studies.

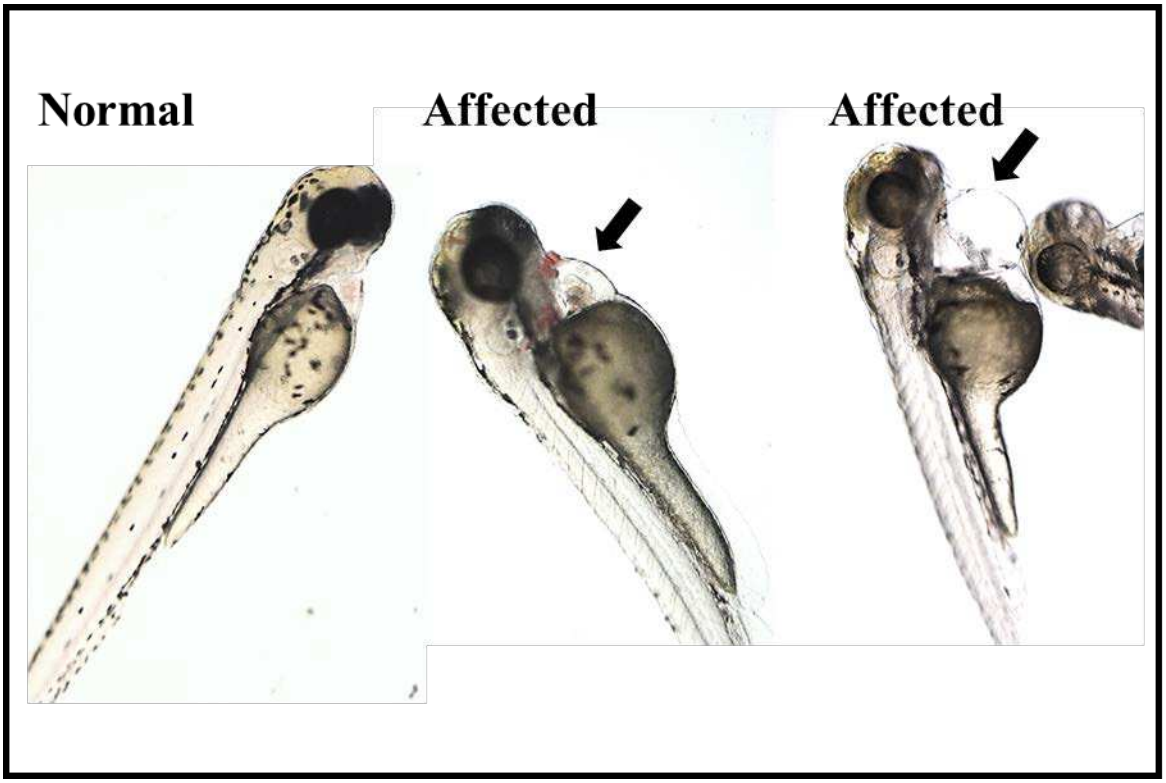


Figure S-3h. Corresponding Morphology from the Individual Studies: Presence of Pericardial Edema. Pictures captured at 40x on Day 3 of the individual studies. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. The anterior region of each embryo is oriented to the top. Disregard embryo perpendicular to the affected on the far right. This observation was statistically significant in the fenofibrate (FENO), fluvastatin (FLUV), and pitavastatin (PIT) drug studies.

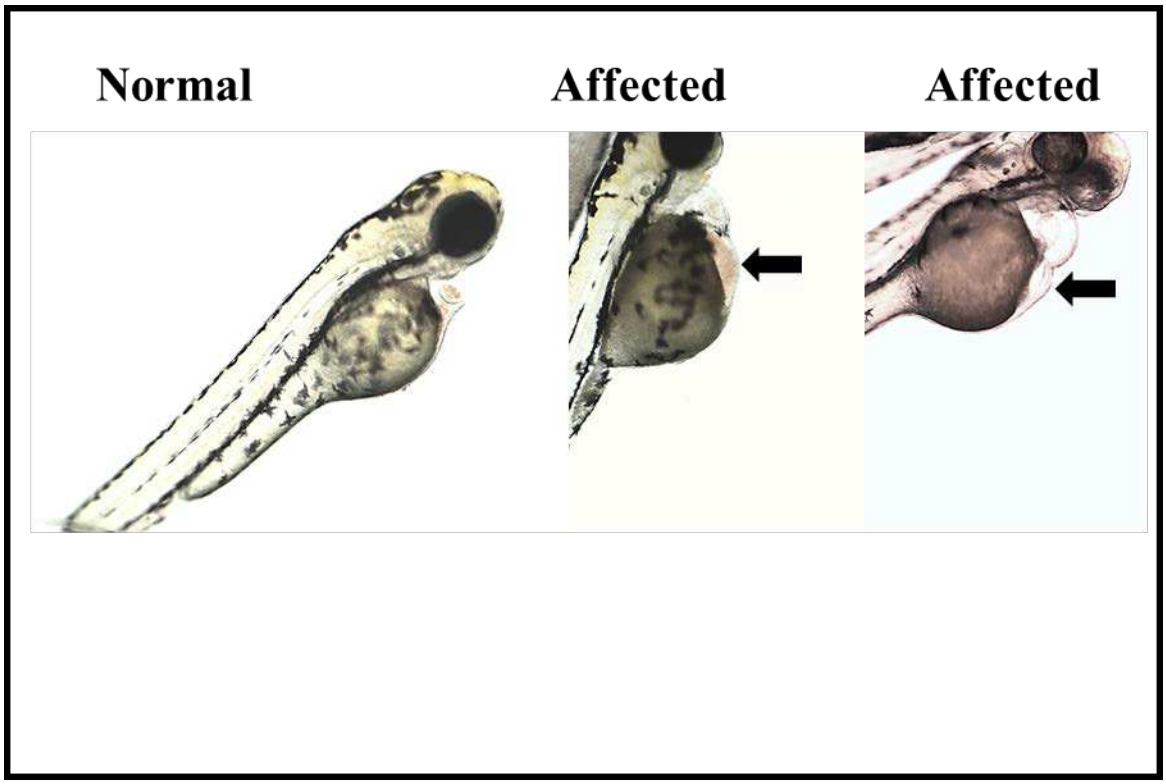


Figure S-3i. Corresponding Morphology from the Individual Studies: Presence of Yolk Edema. Pictures captured at 40x on Day 3 of the individual studies. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. The anterior region of each embryo is oriented to the top or top-right. Disregard embryo to the left of the first affected yolk edema and the embryo directly above the affected on the far right. This observation was statistically significant in the fenofibrate (FENO), fluvastatin (FLUV), and pitavastatin (PIT) drug studies.

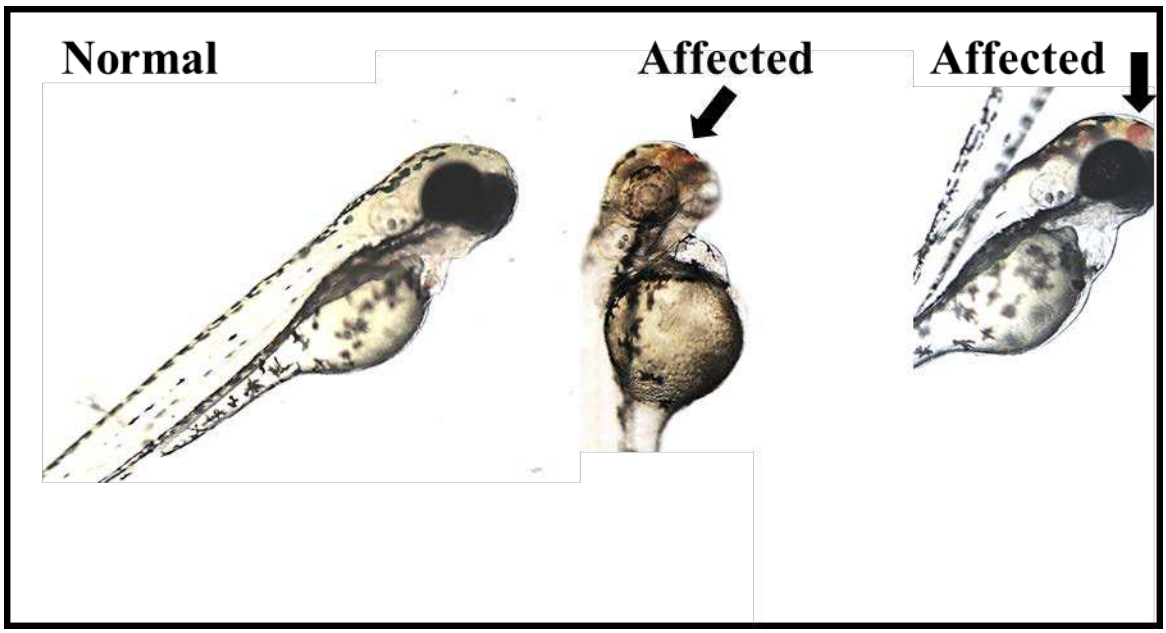


Figure S-3j. Corresponding Morphology from the Individual Studies: Presence of Hemorrhage. Pictures captured at 40x on Day 3 of the individual studies. Examples of morphology evaluated as "Normal" and "Affected" (or abnormal) are displayed. The anterior region of each embryo is oriented to the top or top-right. Disregard embryo perpendicular to and above the affected on the far right. This observation was statistically significant in the atorvastatin (ATO), fluvastatin (FLUV), and pitavastatin (PIT) drug studies.

APPENDIX 4

WATER CHEMISTRY VALUES

Table S-4a

Water Chemistry Values from the Mixture Studies

pH				
Study	Mean	Standard Deviation	Minimum	Maximum
ZF	7.9	0.2	7.3	8.0
FHM	7.9	0.1	7.6	8.2
Dissolved Oxygen				
Study	Mean (mg/L)	Standard Deviation (mg/L)	Minimum (mg/L)	Maximum (mg/L)
ZF	8.9	0.3	8.3	9.6
FHM	8.8	0.1	8.6	9.1
Temperature				
Study	Mean (°C)	Standard Deviation (°C)	Minimum (°C)	Maximum (°C)
ZF	28	1	28	29
FHM	25	1-2*	23*, 24	26

Note. Water chemistry values from ZF and FHM mixture studies from freshly-prepared and day-old exposure solutions. Immediately after test initiation, embryos remained at 23 °C an additional two hours; remainder of the study was 25 ± 1 °C. ZF = zebrafish, FHM = fathead minnows.

Table S-4b

Water Chemistry Values from the Individual Studies

pH

Study	Mean	Standard Deviation	Minimum	Maximum
Fenofibrate (FENO)	7.9	0.1	7.7	8.1
Gemfibrozil (GEM)	7.8	0.1	7.5	8.0
Atorvastatin (ATO)	7.9	0.1	7.6	8.0
Fluvastatin (FLUV)	7.9	0.2	7.4	8.1
Lovastatin (LOV)	7.9	0.1	7.6	8.1
Pitavastatin (PIT)	7.9	0.1	7.7	8.1
Pravastatin (PRAV)	7.9	0.1	7.7	8.1
Rosuvastatin (ROS)	8.0	0.1	7.7	8.1
Simvastatin (SIM)	8.0	0.1	7.6	8.1

Dissolved Oxygen

Study	Mean (mg/L)	Standard Deviation (mg/L)	Minimum (mg/L)	Maximum (mg/L)
Fenofibrate (FENO)	NM	NM	NM	NM
Gemfibrozil (GEM)	8.8	0.1	8.5	9.0
Atorvastatin (ATO)	8.4	0.4	7.6	8.7
Fluvastatin (FLUV)	8.6	0.1	8.5	8.7
Lovastatin (LOV)	8.3	0.2	8.1	8.6
Pitavastatin (PIT)	8.5	0.1	8.5	8.7
Pravastatin (PRAV)	8.8	0.1	8.5	9.1

Rosuvastatin (ROS)	8.4	0.5	7.6	8.8
Simvastatin (SIM)	8.8	0.2	8.4	8.9

Temperature

Study	Mean (°C)	Standard Deviation (°C)	Minimum (°C)	Maximum (°C)
Fenofibrate (FENO)	NM	NM	NM	NM
Gemfibrozil (GEM)	28	0	28	29
Atorvastatin (ATO)	28	1	28	29
Fluvastatin (FLUV)	28	1	26	29
Lovastatin (LOV)	28	0	28	28
Pitavastatin (PIT)	28	0	28	30
Pravastatin (PRAV)	28	1	28	29
Rosuvastatin (ROS)	28	1	27	29
Simvastatin (SIM)	28	1	28	29

Note. Water chemistry values from zebrafish individual studies from freshly-prepared and day-old exposure solutions. NM = not measured.

APPENDIX 5

ANALYTICAL DETAILS

Table S-5a

UPLC-MS/MS Conditions: Part 1

Drug	**ESI Mode**	**$[M+H]^+$ (*m/z*)**	**Product Ion 1 (*m/z*)**	**Product Ion 2 (*m/z*)**	**Retention Time (mins)**
Fenofibrate (FENO)	Positive	361.1	233	138.9	8.15
Gemfibrozil (GEM)	Negative	249.2	120.9	105.7	6.8
Atorvastatin (ATO)	Positive	559.3	440.2	250.1	6.05
Fluvastatin (FLUV)	Positive	412.2	266.1	224.1	5.95
Lovastatin (LOV)	Positive	405.3	285.2	199.1	4.15
Pitavastatin (PIT)	Positive	422.2	318.2	290.2	4.15
Pravastatin (PRAV)	Negative	423.2	321.3	101	3.1
Rosuvastatin (ROS)	Positive	482.2	300.2	258.1	4.4
Simvastatin (SIM)	Positive	419.3	285.2	199.1	7.65

Note. ESI = electrospray ionization; *m/z* = mass-to-charge ratio; mins = minutes.

Table S-5b

UPLC-MS/MS Conditions: Part 2

Drug	Dwell Time (ms)	Fragmentor Voltage (V)	Collision Energy (eV)	Cell Accelerator Voltage (V)
Fenofibrate (FENO)	20	115	12/32	7
Gemfibrozil (GEM)	50	70	8/48	7
Atorvastatin (ATO)	20	140	20/48	7
Fluvastatin (FLUV)	20	100	12/29	7
Lovastatin (LOV)	20	70	4	7
Pitavastatin (PIT)	20	160	32/28	7
Pravastatin (PRAV)	50	100	4/20	7
Rosuvastatin (ROS)	20	150	40/32	7
Simvastatin (SIM)	20	80	4/12	7

Note. Conditions for each drug detected in a single run. The first of two values (time or energy) applied to Product Ion 1 and the second applied to Product Ion 2. One listed value applied to both product ions. Product ions listed in Table S-5a. ms = milliseconds; V = volts; eV = electron volts.

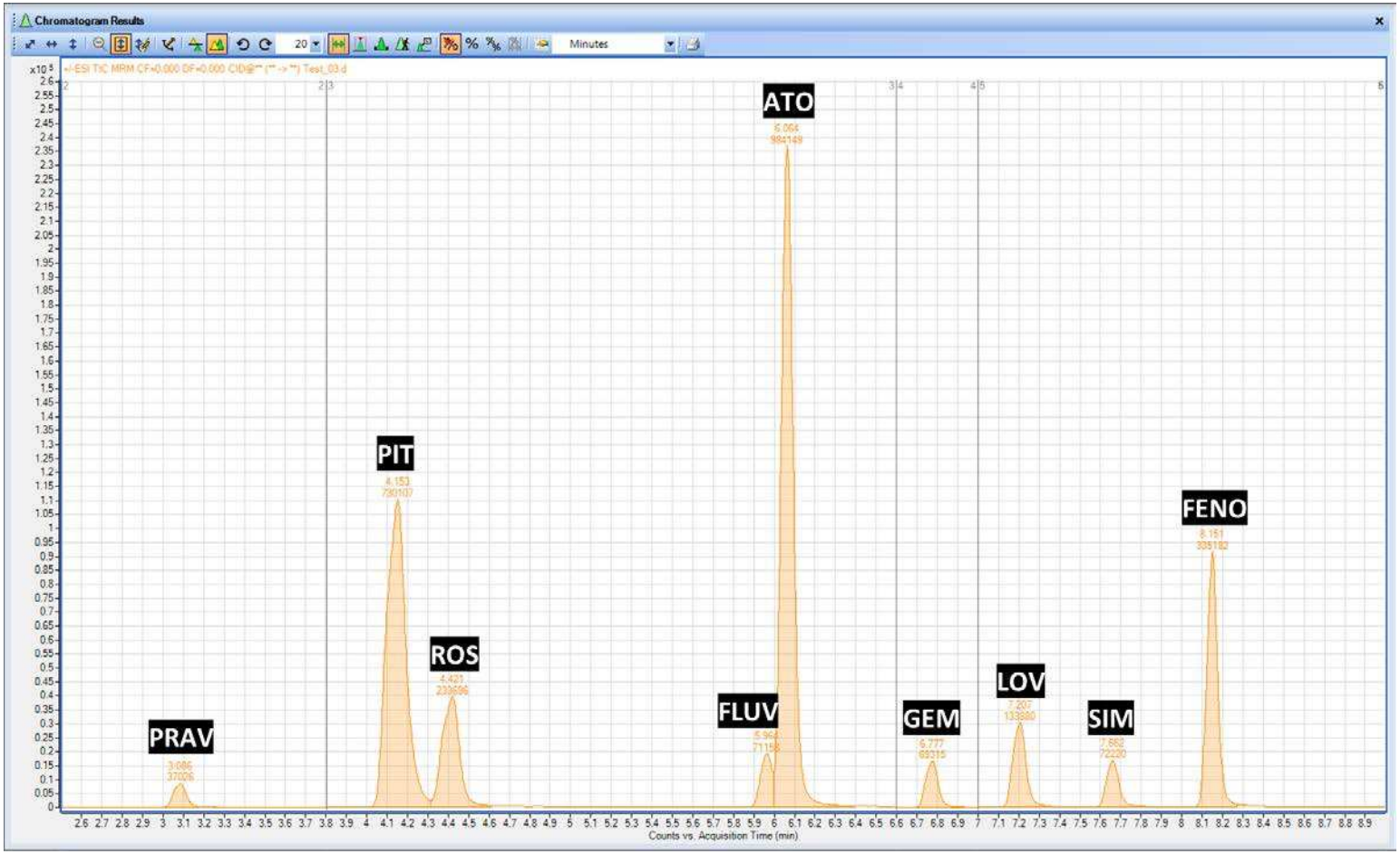


Figure S-5a. UPLC-MS/MS Chromatogram Total Ion Counts. The total ion counts for each drug detected in one run are displayed.

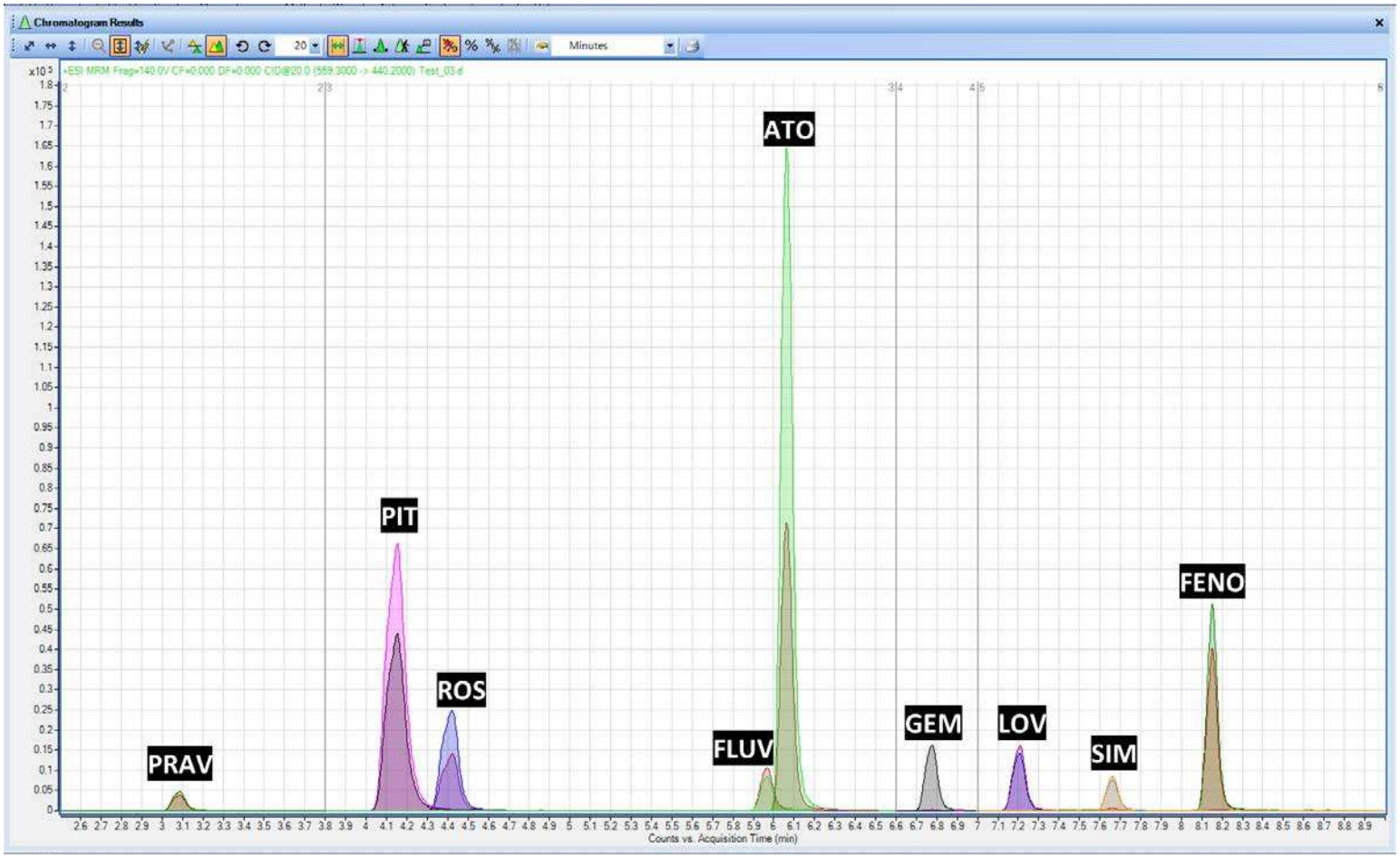


Figure S-5b. UPLC-MS/MS Chromatogram MRM. Two product ions for each drug are visually displayed from one run.

APPENDIX 6

FATHEAD MINNOW UNPUBLISHED STUDY

A 28-day preliminary laboratory study with exposure of gemfibrozil in fathead minnow (*Pimephales promelas*) larvae was performed. Two nominal exposure concentrations (0.5 or 2.2 mg GEM/L) were selected and impacts to growth and length were determined. An overview of this study's methods and results were previously published (Cadmus & Jefferson, 2016). No significant effects were determined based on overlapping means and standard deviations (Table S-6).

Table S-6

Results from a 28-day Exposure of Fathead Minnows to GEM

Concentration (mg GEM /L)	Standard Length (mm)	Total Length	Blot Wet Weight (g)	Dry Weight (g)
0 (DMSO only)	24.1 (1.5)	29.1 (1.7)	0.20 (0.04)	0.04 (0.01)
0.5	23.7 (1.7)	28.5 (2.0)	0.19 (0.04)	0.04 (0.01)
2.2	23.6 (2.2)	28.0 (3.0)	0.19 (0.05)	0.04 (0.00)

Note. Fish were 60 days post hatch at study termination when these measurements were taken. One standard deviation is shown in parentheses. No significant differences were observed. Concentrations are nominal.

APPENDIX 7

CONCENTRATIONS DETECTED IN THE ENVIRONMENT

Table S-7

Environmentally-Relevant Concentrations by Drug from Previous Research

FENO

Influent (ng/L)	Effluent (ng/L)	Surface Water (ng/L)	Ground-water (ng/L)	Drinking Water (ng/L)	Location	Author
--	ND-20-160	--	--	--	Europe	(Andreozzi, Marotta, & Paxeus, 2003)
ND[d]; --	--; ND	--	--	--	Canada; USA	(Carrara et al., 2008) (Drewes, Heberer, Rauch, & Reddersen, 2003)
--	--	ND-1.23	--	--	Spain	(da Silva et al., 2011)
ND	ND	ND-9	--	--	Spain	(M. Gros, Petrovic, & Barcelo, 2009)
--	ND-30-160; 120[a]	--	--	--	Europe & Canada	(Hernando, Aguera, & Fernandez-Alba, 2007)(R)
1-300	1-2,353	0.6-61	0.4-27	--	--	(Rezka & Balcerzak, 2015)(R)
--	--	--; 30	--	--	USA; EU	(Sanderson, Johnson, Wilson, Brain, & Solomon, 2003)(R)
--	30[b]	ND	--	--	Germany	(Ternes, 1998)

GEM

Influent (ng/L)	Effluent (ng/L)	Surface Water (ng/L)	Ground-water (ng/L)	Drinking Water (ng/L)	Location	Author
--	60-4,760; 840[a]	--	--	--	Europe	(Andreozzi et al., 2003)
--	--	32.3-50.9[a]; 409-677[b]	--	--	USA (CO)	(Bai et al., 2018)
710	180	--	--	--	Sweden	(Bendz, Paxeus, Ginn, & Loge, 2005)

15-620[d]	--	--	--	--	Canada	(Carrara et al., 2008)
--	--	12-200	--	--	Canada	(Csiszar et al., 2011)
--	1,235	--	--	--	USA	(Drewes et al., 2003)
--	--	7.3	--	--	USA (CO, UT)	(EPA 2015)*
3,470-63,800	80-19,400	--	ND-110-6,860	--	USA (TX)	(Fang et al., 2012)
--	--	ND-0.91-212	--	--	Spain	(da Silva et al., 2011)
--	59-84; 71[a]	--	--	--	Canada	(Gagné, Blaise, & Andre, 2006)
--	1,420	--	--	--	Spain	(Ginebreda et al., 2010)
--	ND-320	ND-60	--	--	Spain	(M. Gros, Petrovic, & Barcelo, 2006)
26-1,040	2-378	0.7-76	--	--	Spain	(M. Gros et al., 2009)
924-5,120	65-1,018	12-284	--	8	Spain	(Meritxell Gros, Rodriguez-Mozaz, & Barcelo, 2012)
ND	--	ND	--	--	South Korea	(Han, Hur, & Kim, 2006)
--	--	ND-35	--	--	Germany	(Heberer, Reddersen, & Mechlinski, 2002)
--	70	--	ND-340	--	Germany	(Heberer, 2002)(R)
--	ND-0.84-4,760; 7-1,510[a]	ND-1-1,550; 48-250[a]	--	--	Europe & Canada	(Hernando et al., 2007)(R)
--	--	76.9-286	--	--	Spain	(Huerta et al., 2016)
--	--	22	--	--	Germany	(Jux, Baginski, Arnold, Kronke, & Seng, 2002)
--	--	790[b]; 48[c]	--	--	USA	(Kolpin et al., 2002)
--	--	ND-5-14	--	--	Canada	(Li, Helm, & Metcalfe, 2010)
--	--	ND-60.4-113	--	--	Spain	(Lopez-Serna, Petrovic, & Barcelo, 2012)
2,100[b]; 700[c]	1,300	--	--	--	Canada	(Metcalfe, Koenig, et al., 2003)
--	5-1,493	--	--	--	Canada	(Metcalfe, Miao, Koenig, & Struger, 2003)
--	--	11-70	--	--	Spain	(Osorio, Larranaga, Acena,

						Perez, & Barcelo, 2016)
--	9-300	5.4-16	--	--	North America	(Pal, Gin, Lin, & Reinhard, 2010)R)
--	2-28,571; 3.9-17	--; 1.9-9.1	--	--	Europe; Asia/Australia	(Pal et al., 2010)R)
--	60-870; 560[c]	--	--	--	Europe	(Paxeus, 2004)
--	--	30.1-73.9; 54.3[a]	--	--	Spain	(Radjenovic, Petrovic, & Barcelo, 2007)
100-17,100	1-6,050	1-17,036	12-574	--	--	(Rezka & Balcerzak, 2015)(R)
--	--	~200-300	--	--	Europe	(Ruhi et al., 2016)
--	--	790; 1,500	--	--	USA; EU	(Sanderson et al., 2003)(R)
--	70[b]	--	--	--	Canada	(Tauber, 2003)
--	1,500[b]	510; ND-20-30	--	--	Germany	(Ternes, 1998)
4,770	9	170	--	<0.25	Nevada	(Vanderford & Snyder, 2006)
--	--	<2-27	--	--	Europe	(Wiegel et al., 2004)

ATO

Influent (ng/L)	Effluent (ng/L)	Surface Water (ng/L)	Ground-water (ng/L)	Drinking Water (ng/L)	Location	Author
--	--	6.8[e]	--	--	USA (TN, NC, VA)	(Conley, Symes, Schorr, & Richards, 2008)
--	--	7.3	--	--	USA	(Deo, 2014)(R)
--	ND-16-128	8-35	--	--	Italy	(Ferrari et al., 2011)
--	--	ND-5.58-52.3	--	--	Spain	(da Silva et al., 2011)
25-180	9-395	ND-5	--	--	Spain	(M. Gros et al., 2009)
153-182	8-111	1-3	--	1	Spain	(Meritxell Gros et al., 2012)
--	37; 22.4[a]	ND-1	--	--	Europe & Canada	(Hernando et al., 2007)(R)
ND-23.3	ND	--	--	--	Slovenia	(Klancar, Trontelj, Kristl, Justin, & Roskar, 2016)
72-263; 166[c]	10-122; 77[c]	--	--	--	Canada	(Lee, Peart, Svoboda, & Backus, 2009)
--	ND-19-44		--	--	Canada	(Metcalfe, Miao, et al., 2003)

76	37	1	--	--	Canada	(Miao & Metcalfe, 2003)
1-18	1-6	0.25-1.4	--	--	--	(Rezka & Balcerzak, 2015)(R)
201	<0.5	7.3	--	<0.25	USA (NV)	(Vanderford & Snyder, 2006)

FLUV

Influent (ng/L)	Effluent (ng/L)	Surface Water (ng/L)	Ground-water (ng/L)	Drinking Water (ng/L)	Location	Author
ND-43	ND-12	ND	--	ND	Spain	(Meritxell Gros et al., 2012)
--	--	ND	--	--	Spain	(Huerta et al., 2016)

LOV

Influent (ng/L)	Effluent (ng/L)	Surface Water (ng/L)	Ground-water (ng/L)	Drinking Water (ng/L)	Location	Author
--	10	ND	--	--	Malaysia	(Al-Odaini, Zakaria, Yaziz, & Surif, 2010)
--	--	18.3[e]	--	--	USA (TN, NC, VA)	(Conley et al., 2008)
--	14	ND	--	--	Europe & Canada	(Hernando et al., 2007)(R)
49	14	ND	--	--	Canada	(Miao & Metcalfe, 2003)

PIT

Influent (ng/L)	Effluent (ng/L)	Surface Water (ng/L)	Ground-water (ng/L)	Drinking Water (ng/L)	Location	Author
--	--	--	480	--	India	(Jindal, Narayanam, & Singh, 2015)

PRAV

Influent (ng/L)	Effluent (ng/L)	Surface Water (ng/L)	Ground-water (ng/L)	Drinking Water (ng/L)	Location	Author
--	--	7.3	--	--	USA (CO, UT)	(EPA 2015)*
--	--	1.18-14.5	--	--	Spain	(da Silva et al., 2011)
-- ND --	ND ND ND	-- ND ND	--	--	Spain Spain Spain	(Ginebreda et al., 2010) (M. Gros et al., 2006)

						(M. Gros et al., 2009)
ND-152	ND-36	ND	--	ND	Spain	(Meritxell Gros et al., 2012)
--	59	ND	--	--	Europe & Canada	(Hernando et al., 2007)(R)
--	--	13.8-44	--	--	Spain	(Huerta et al., 2016)
--	--	ND-0.07-7.73	--	--	Spain	(Lopez-Serna et al., 2012)
117	59	ND	--	--	Canada	(Miao & Metcalfe, 2003)
--	--	ND	--	--	Spain	(Radjenovic et al., 2007)
50-1,080	3-70	1.6-5	--	--	--	(Rezka & Balcerzak, 2015)(R)
--	--	1.6	0	5[b]	France	(Vulliet & Cren-Olive, 2011)

ROS

Influent (ng/L)	Effluent (ng/L)	Surface Water (ng/L)	Ground-water (ng/L)	Drinking Water (ng/L)	Location	Author
134-604; 448[c]	190-552; 324[c]	--	--	--	Canada	(Lee et al., 2009)

SIM

Influent (ng/L)	Effluent (ng/L)	Surface Water (ng/L)	Ground-water (ng/L)	Drinking Water (ng/L)	Location	Author
--	ND	ND	--	--	Malaysia	(Al-Odaini et al., 2010)
--	--	0.74[f]	--	--	USA	(Deo, 2014)(R)
--	1	ND	--	--	Europe & Canada	(Hernando et al., 2007)(R)
4	1	ND	--	--	Canada	(Miao & Metcalfe, 2003)
1-115	1-5	--	--	--	--	(Rezka & Balcerzak, 2015)(R)
<2.5	<0.5	<0.25	--	<0.25	USA (NV)	(Vanderford & Snyder, 2006)
10[f]	<0.5[f]	0.74[f]	--	<0.25[f]	USA (NV)	(Vanderford & Snyder, 2006)

Note. Concentrations detected in the environment from previous studies are reviewed; surface water included concentrations from rivers, streams, ponds, lakes, and/or reservoirs; R = review article; values may be reviewed separately or from a review article; *unpublished data-samples collected from variety of locations primarily in Colorado during 2015. [a] mean concentration, [b] maximum concentration, [c] median concentration, [d] from septic tank, [e] value summarized from effluent and surface water samples, [f] hydroxy acid form, ND = not detected, below limit of detection, below limit of quantification, "—" = not measured. Refer to Appendix 8 for these specific references.

APPENDIX 8

REFERENCES FOR SUPPLEMENTAL INFORMATION

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LIST OF ABBREVIATIONS

AP - anterior-posterior (axis)

ATO – Atorvastatin

dpf – days post fertilization

DMSO - dimethyl sulfoxide

ERC – environmentally-relevant concentration

FENO - Fenofibrate

FLUV - Fluvastatin

GEM - Gemfibrozil

GFP - green fluorescent protein

hpf - hours post fertilization

ISV - intersegmental vessels

LOV - Lovastatin

NM - not measured

NT - non transgenic

PIT - Pitavastatin

PRAV - Pravastatin

ROS - Rosuvastatin

SIM - Simvastatin

SIV - subintestinal veins

T - transgenic (green fluorescent protein (GFP) expressed)