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Three Generational Exposure of Pimephales promelas to an Urban Contaminants of Emerging Concern Mixture

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Three Generational Exposure of *Pimephales promelas* to an

Urban Contaminants of Emerging Concern Mixture

by

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Abstract

Complex mixtures of contaminants of emerging concerns (CECs) are present in many Great Lakes tributaries. Using existing chemical occurrence and concentration data for CECs from water samples collected in streams influenced by urban land use, the current study assessed how an urban-derived CEC mixture affected three generations of fathead minnows (*Pimephales promelas*) exposed continuously under controlled laboratory conditions. Mature and larval minnows were exposed to a CEC mixture comprised of: Galaxolide (synthetic musk), TBEP (plasticizer), Estrone, Bisphenol-A (plasticizer), DEET, Methyl-1H-benzotriazole (anti-icing agent), Desvenlafaxine (anti-depressant), Fexofenadine (allergy medication), Metformin (diabetes medication), and Nonylphenol. The medium mixture contained all of the above compounds at their highest environmentally measured concentrations, while the low mixture used $1/10^{th}$ and the high mixture 10x that concentrations. F1 fish were exposed while sexually mature and produced F2 generation *P. promelas*, which were then exposed throughout their entire life cycle. F3 generation fish were exposed until sexual differentiation. A multitude of biological endpoints were measured to assess the effects of the urban CEC mixture on fish health and development. F1 minnows exposed to the urban mixture had higher plasma vitellogenin concentrations than control fish (mean: 2.73 ug/mL and 1.91 ug/mL respectively, ANOVA, $p < 0.05$). Exposure did not have a significant effect on body condition factor, gonadal somatic index, or hepatic somatic index. F2 larvae had a faster response to a predator stimulus than F3 larvae ($p=0.0013$). F2 larvae exposed to the high concentration were also larger (ANOVA, $p = 0.01$). F1 and F2 fecundity was greater in the low and medium treatments than those exposed to the high and EtOH control (Repeated-measures ANOVA, $p<0.0001$) suggesting a therapeutic hazard originating from the CEC mixture exposure that contained numerous pharmaceutical compounds. The alteration in apical endpoints central to sustaining fish populations suggests that complex urban CEC mixtures can pose a therapeutic hazard to fish populations.

Acknowledgements

For my grandfather

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CHAPTER 1: LITERATURE REVIEW

1.1 Contaminants of Emerging Concern: Effects on Fish Physiology

Contaminants of emerging concern (CECs) are naturally or synthetically derived chemicals, seldom monitored by environmental agencies, that cause known or suspected adverse health effects (Megdal, 2013; Ankley et al., 2008; Ankley et al., 2005). In some cases, CECs are also found to be endocrine disrupting compounds (EDCs), which mimic hormones and disrupt normal endocrine function (Megdal, 2013; Sonnenschein & Soto, 1998). Apart from interacting with hormone receptors, endocrine disrupting CECs can affect the function of the hypothalamic-pituitary-gonadal (HPG) axis in vertebrates (Ankley et al., 2005; Kazeto et al., 2004). Furthermore, laboratory studies with small fish have highlighted developmental and reproductive consequences of CEC exposure (as reviewed in Boxall et al., 2012). Some CECs such as Bisphenol-A have been found to mimic both estrogen and androgens, leading to the hypothesis that some CECs may have multiple hormonal properties (Mills & Chichester, 2005).

In female *Pimephales promelas* (common name: fathead minnow), gonadotrophin-releasing hormone is secreted to the pituitary gland in the hypothalamus, signaling the release of luteinizing and follicle-stimulating hormones (Hadley & Levine, 2006). These hormones facilitate ovarian cells to release androgens and aromatase production, respectively (Hadley & Levine, 2006). Aromatase converts androgens into estrogens, which is responsible for oocyte development and hepatic secretion of vitellogenin (Hadley & Levine, 2006; Jensen et al., 2001). Vitellogenin, an egg-yolk precursor protein, is stored in growing oocytes to serve as a food reserve for developing embryos (Sumpter & Jobling, 1995). Unlike female *P. promelas,* whose plasma vitellogenin concentrations rise during sexual maturation, male *P. promelas* have a silent vitellogenin gene (Sumpter & Jobling, 1995). However, when activated by estrogenic CECs, plasma vitellogenin concentrations will rise in male fish, leading to the use of plasma vitellogenin as an assessment of estrogen exposure in male fish (Panter et al., 1998; Sumpter & Jobling, 1995).

1.2 Biology of the Fathead Minnow, *Pimephales promelas*

P. promelas is a teleost fish belonging to the family Cyprinidae (Rafinesque, 1820). *P. promelas* range from an average of 41 to 71 mm in length with males generally growing faster and larger than females (Becker, 1983; Pflieger, 1975). Both sexes are deep-bodied, with a single, soft-rayed dorsal fin, slightly forked homocercal caudal fin with a dark spot at the base, a blunt head, and a small, subterminal mouth (Nelson & Paetz, 1992). During breeding season, *P. promelas* exhibit strong sexual dimorphism (secondary sex characteristics) with males developing three rows of tubercles on the snout, a thick dorsal pad, and broad, black vertical bands (Smith, 1978). The normal dorsal color ranges from a pale brown or yellowish-olive color with a silvery toned lateral body (Figure 1; Pflieger, 1975). The species is widely distributed throughout North America and has been introduced beyond its native range as a result of its prominent use as a baitfish among anglers (Moyle, 1976; Smith-Vaniz, 1968). *P. promelas* are omnivorous feeders that dwell in a variety of habitats including small ponds, streams, lakes, and slow creeks with different substrates including sand, silt, rubble, and silt (Becker, 1983; Smith, 1978; Pflieger, 1975). Despite its broad range, it has been observed that *P. promelas* is very intolerant of competition and predation, therefore being seldom seen in habitats with high fish diversity (Pflieger, 1975). *P. promelas* can tolerate temperatures up to 33°C (Moyle, 1976), high turbidity (average of 77NTU; Trebitz et al., 2007), periods of low oxygen (<5.0 mg/L; Brungs, 1971), and a range of pH levels (4-9.1) (Mount, 1973; McCarraher & Robert, 1968), making them a highly successful cyprinid species that plays an important role in the structure and function of aquatic ecosystems by influencing the food web (Nelson & Paetz, 1992; Becker, 1983). Due to its wide range of distribution, water quality tolerance, and well-defined reproductive cycle and behavior, it is prevalent as a laboratory model fish for various types of experiments including, behavioral, toxicity, and regulatory purposes (USEPA, 1987).

Figure 1. A male *P. promelas* during mating season exhibits secondary sexual characteristics including

tubercles on the snout, dorsal pad anterior to the dorsal fin, and vertical banding.

As a fractional spawner, *P. promelas* can spawn continuously under suitable conditions beginning

in mid-April, when temperatures reach approximately 15.6°C (60°F), through most of the summer months

into the fall, when the temperatures drop below this threshold (Danylchuk & Tonn, 2001; Duda, 1989;

Becker, 1983). Males begin to develop secondary sex characteristics 30 days prior to spawning (Markus,

1934). Male *P. promelas* exhibit distinct, territorial behavior during the breeding season (Divino & Tonn,

2008), which often regards them as one of the most defensive and egg attentive freshwater species (Smith,

1978). Once the development of secondary sex characteristics begins, male *P. promelas* find a relatively

flat substrate (i.e. woody debris, rock overhang) as his nesting site before attempting to attract females

and driving away other males (Divino & Tonn, 2008; Becker, 1983; Unger, 1983). Females lay an

average of 400-500 eggs per clutch every three to four days (Divino & Tonn, 2008). The male then guards

the eggs aggressively, using his tubercles to drive away intruders whilst the spongy dorsal pad is used to

keep the eggs clean and agitated to create flow around the eggs until they hatch in approximately four to

five days (Divino & Tonn, 2008; Ankley & Villenueve, 2006; Unger, 1983). To minimize fungal

infections, the male will occasionally remove infected eggs by nibbling the clutch to prevent the fungus

from spreading (McMillian, 1972). Once the larvae have hatched, they will remain around the nest site

until their entire yolk-sac is absorbed (Becker, 1983). Larvae grow rapidly and are an average of 4.75 mm

at hatching (Markus, 1934). At 20 days and 60-days post-hatch, larvae average 20 mm and 29 mm in length respectively (Becker, 1983). Within four to five-months post-hatch, *P. promelas* are reproductively mature, if conditions are suitable (Ankley & Villeneuve, 2006).

1.3 Mixture Exposure of Fish

In the environment, fish are exposed to mixtures of chemicals from various sources. In these complex mixtures, there are pharmaceuticals, industrial byproducts, personal care products, and even industrial chemicals such as anti-icing agents present. A complex mixture is defined as a mixture with multiple components with diverse modes-of-action in which any estimation of the individual chemicals' toxicities has too much uncertainty and error to be useful (USEPA, 2000). The individual chemicals can differ in composition overtime (i.e. one chemical is 50% of the mixture one day and 20% the next month) depending on the time and conditions the complex mixture is created in the environment (USEPA, 2000). A simple mixture, as defined by the USEPA (2000), is "a mixture containing two or more identifiable components, but few enough that the mixture toxicity can be adequately characterized by a combination of the components' toxicities and the components' interactions" (USEPA, 2000, p. B-2).

Although single chemical exposure effects are easier to determine, it is unknown whether chemicals in a mixture act in a synergistic, antagonistic, or additive nature. Synergism and antagonism, defined by "no interaction" for the endpoint (USEPA, 2000), is suggested when the effect of a mixture (two or more chemicals) is greater or less than the sum of individual chemical effects, respectively (Cedergreen, 2014; Preston et al., 2000; USEPA, 2000). Synergism can be calculated or predicted from the Concentration Addition model ($\frac{A}{A_1} + \frac{B}{B_1} = 1$ where A and B are the chemical concentrations and A1 and B1 are the lowest observable effects concentration) by Bliss (1939). This model uses mortality and the assumption that chemicals act similarly in organisms to predict mixture toxicity (Bliss, 1939).

Additivity is suggested when the effect of a mixture is equal to the sum of effects of individual chemicals when assessed separately (Preston et al., 2000; USEPA, 2000).

In one instance a single chemical exposure to a pharmaceutical was found to increase female fecundity, but to also decrease fish sociality (Klaminder et al., 2014; Brodin et al., 2013). In another case where two antagonizing chemicals (17ß-estradiol and nonylphenol) were used in an exposure of zebrafish, the researchers found the two chemicals behaved additively and non-additively, in reference to vitellogenin induction, depending on the concentration of the two chemicals used (Lin & Janz, 2006). Japanese medaka exposed to different mixtures of E2, tamoxifen, and letrozole had adverse effects on fecundity, hepatic somatic index, and fertility (Sun, Zha, & Wang, 2009). Exposure of E2 and tamoxifen was found to impair fecundity and vitellogenin values more severely (synergistic), while exposure to E2 and letrozole were weakly antagonistic, but E2 effects were too strong for letrozole to completely antagonize (Sun, Zha, & Wang, 2009). Jukosky et al. (2007) exposed adult medaka to environmentally relevant concentrations of 17α-estradiol (EE2), E2, and nonylphenol (simple mixtures) for 14 days. The researchers found lower vitellogenin concentrations in fish exposed to the mixture than those exposed to E2 (Jukosky et al., 2007). They suggested relative estogenicity (additive mixture models) of compounds is useful for predicting some mixture effects (specifically for reproductive), but not for all biological endpoints (Jukosky et al., 2007).

As suggested by the studies of Juskosky et al. (2007), effects of mixture exposures are not easily characterized. A study by Filby et al. (2007) characterized the effects of two wastewater treatment plant effluents (potent vs weak estrogenic mixtures) on *P. promelas* for 21 days. Filby et al. (2007) found no effects on weight, length, body condition factor, hepatic expression of igf1, or gonadal somatic index. However, the researchers found secondary sex characteristics in male *P. promelas* were reduced when exposed to the potent effluent mixture (Filby et al., 2007). Vitellogenin concentrations were higher in both potent and weak effluent exposures compared to the control (Filby et al., 2007). Schoenfuss et al.

(2016) exposed larval and adult *P. promelas* to simple and complex mixtures of hydrocodone, methadone, oxycodone, tramadol, methocarbamol, fluoxetine, paroxetine, venlafaxine, and temazepam and compared them to exposure done with wastewater effluent. The researchers found no effects of the mixtures or effluent on larvae (Schoenfuss et al., 2016). However, when adult male *P. promelas* were assessed for vitellogenin concentrations, those exposed to the effluent had higher concentrations (Schoenfuss et al. 2016). In addition, exposure to the different pharmaceutical mixtures caused reduced nest defense activity in male *P. promelas* (Schoenfuss et al., 2016). In a study by Brian *et al*. (2007), *P. promelas* were exposed to single estrogenic chemicals (17ß-estradiol, 17α-ethynylestradiol, 4-*tert*-nonylphenol, 4-*tert*-octylphenol, BPA) and two mixtures types of the estrogenic chemicals (concentration-response and low-dose). Brian et al. (2007) found an increased sensitivity to the chemicals in the males, leading to reduction in tubercle number when exposed to a concentration-response based mixture. Both mixtures documented lower fecundity and induced vitellogenin concentrations in males compared to any other treatment groups (i.e. single chemical exposures), leading the authors to believe the effects indicated a synergistic response (Brian et al., 2007). Other studies have correlated CEC mixture exposure with intersex in white suckers (Vadja et al. 2008), rainbow darters (Hicks et al., 2017), and smallmouth bass (Blazer et al., 2014). These studies exemplify how difficult it is to determine the biological effects of CECs on fish and whether CECs act synergistically, antagonistically, or additively in a mixture (both simple and complex).

1.4 Exposure Length

The US Environmental Protection Agency (2011) defines general acute exposures as "exposure by the oral, dermal, or inhalation route for 24 hours or less" and chronic exposures as "Repeated exposure by the oral, dermal, or inhalation route for more than approximately 10% of the life span in humans (more than approximately 90 days to 2 years in typically used laboratory animal species)". Some short-term (<21 days) exposures using various fish species, such as zebrafish, Japanese medaka, *P. promelas*, and Chinook salmon, characterizing CEC effects, as single and mixture exposures, have found delayed effects

on reproductive events due to changes in sex determination (Cripe et al., 2010; Nash ,2004), egg

production (Nash, 2004), and phenotypical changes (Cripe et al., 2010). This delayed effect could lead to

a potential mischaracterization of the chronic effects CECs have on fish species (Parrot et al., 2017;

Overturf et al., 2015). For example, Nash et al. (2004) found exposure of zebrafish to EE2 at the larval

stage impacted gonadal differentiation leading to decreased reproductive success at the adult stage (Nash,

2004). Chronic exposure (six weeks) of zebrafish to wastewater and a pharmaceutical mixture found

adverse effects on oocyte development and kidney proximal tubule morphology (Galus et al., 2013). Fish

exposed to estrogens and androgens seem to have a different response in acute and chronic exposures. In

species such as *P. promelas* (Kidd et al. 2007), zebrafish (Galus et al., 2013; Nash et al., 2004), and

Japanese medaka (Cripe et al., 2010), there appears to be a slight decline of fecundity in the parental

generation, but an even more pronounced decrease in fecundity in subsequent generations, sometimes

leading to cessation of viable offspring.

In multigenerational exposures (direct exposure of an organism over multiple generations,

Skinner 2008), the most common adverse outcome is a decrease in fecundity (Cripe et al., 2010;

Raimondo et al., 2009). Kidd et al. (2007), conducted one of the most publicized exposures of *P.

promelas* using a synthetic estrogen. The researchers examined a population of *P. promelas* in the

Experimental Lakes Area in Ontario, Canada over a course of seven years. At the end of the three-year

exposure, they found the entire *P. promelas* population collapsed due to the feminization of males. It

should be noted that the biological effects of CEC exposure should be similar between generations (Parrot

et al., 2017). Similar biological effects of CEC exposure, but increased sensitivity was seen by Cripe et

al. (2010) when three generations of sheepshead minnows were exposed to the androgen, 17ß-

Trenbolone. The authors found increasing adverse impacts on reproduction (cumulative reproduction,

daily egg production, and abnormal egg production) for all three generations, finding an interaction effect

between treatment and generation. In addition, to reproductive effects, all first-generation fish were phenotypically male (Cripe et al., 2010).

The findings of Cripe et al. (2010) and Kidd et al. (2007) show how exposure of the parental generation ultimately affects subsequent generations, regardless of whether they are exposed or not. These results provide the rationale for testing the hypothesis that fish exposed over subsequent generations experience increased sensitivity to the chemicals (observable effects at subsequent lower doses) due to the exposure of eggs during embryogenesis (Parrot et al., 2017).

1.5 Larval Behavior

Predator avoidance in fish species encompasses many different endpoints. One of the most widely studied aspects of predator avoidance is known as a startle response or C-start (Eaton & Emberley ,1991). C-starts are regulated by auditory, visual, and vibrational cues, spurring the activation of Mauthner cells located in the hindbrain (Eaton & Emberley ,1991). There are two different stages to the C-start behavior: stage 1 and stage 2 (Nissomov & Eaton, 1989). Stage 1 (escape angle) consists of the bending of the fish into a letter *C* shape when viewed from above, while stage 2 is when the fish begins to accelerate away from the stimulus (Nissomov & Eaton, 1989). After these two stages, the fish then swims away. Stage 1 is thought to be important since it is determined by the center of mass where predators generally strike (Nissamov & Eaton, 1989). The Mauthner cells are thought to be one component of a network of signals in the c-start behavior pathway (Eaten & Emberley, 1991). The Mauthner cell signaling is hormone responsive, indicating that CEC exposure can disrupt communication of the receptors and motor systems, resulting in a change in behavioral performance (Little & Brewer, 2001).

Previous studies of juvenile zebrafish found adverse effects on locomotion behavior (total swimming distance and time active) when exposed to the flame retardant, 2,2'4,4'-tetrabromodiphenyl ether (Chou et al., 2010). Other studies have found CEC exposure results in delays of visual signal

processing (Wibe et al., 2001), disruption of swimming performance and activity of juvenile fishes (Brown et al., 1985; Zhou & Weis, 1998; Chou et al., 2010). Exposure to heavy metals (copper, cadmium) and herbicides (atrazine and diuron) caused a decreased olfactory response in juvenile rainbow trout (Scott et al., 2003), Colorado pike minnow (Beyers & Farmer, 2001), and goldfish, respectively (Sagle & Trijasse, 1998). Short-term exposure of larval *P. promelas* to environmental concentrations of estrogenic CECs found impaired larval escape performance (McGee et al., 2009), while exposure to Venlafaxine and an antidepressant mixture (Fluoxetine, Sertraline, Venlafaxine, Bupropion) adversely affected total escape response (Painter et al., 2009). These studies show how adverse effects of swimming behavior and c-starts in larval exposure to CECs, heavy metals, and persistent organic pollutants could ultimately lead to a decline in survival in teleost fishes.

Changes in foraging behavior, which can be an indicator of environmental stress (i.e. temperature or CEC exposure) in fish (Feininger, 1990), may also be affected by some CEC exposures. Brodin et al. (2013), exposed European perch to oxazepam, an anxiolytic drug for seven days. In this study, the authors found an increased feeding rate in the exposed fish (Brodin et al., 2013). European perch exposed to 910ug/L of oxazepam began foraging earlier and depleted *Daphnia pulex* faster than those exposed to 1.8 ug/L of oxazepam and unexposed European perch (Brodin et al., 2013). Another study by Bell (2014) found increased growth, increased activity, and increased risky foraging behavior in threespine stickleback exposed to ethinyl estradiol. Despite these seemingly positive effects on the stickleback, survival was lower in exposed fish (Bell, 2014). Additionally, the increase in risky foraging behavior would make the stickleback more susceptible to predation (Bell, 2014).

A different study, found larval largemouth bass exposed to pentachlorphenol for eight weeks post-hatch had decreased brine shrimp predation attempts (Brown et al., 1985). Hybrid striped bass exposed to the antidepressant Fluoxetine (Gaworecki & Klain, 2008) were quicker to eat prey as brain serotonin concentrations increased (Gaworekci & Klaine, 2008). Another exposure of hybrid striped bass

to the antidepressant Venlafaxine found exposed bass took longer to capture prey (Bisesi Jr, Bridges, & Klaine, 2014).

Although there are many factors besides CEC exposure that may influence foraging behavior (i.e. temperature, stress, bioavailability), the studies mentioned above, particularly Brodin et al. (2013) and Bell (2014), demonstrate that low concentration CEC exposure may have a two-sided effect on fish (therapeutic and adverse) depending on the CEC's mode of action.

Adverse effects of CECs, such as those found in Kidd et al. (2007) on *P. promealas*, a key prey species (Becker, 1983), could lead to irreversible food web effects, such as a bottom-up cascade where the prey influence higher tropic forms (Estes, 1995). Understanding how CECs interact with each other to effect biological endpoints such as survival, female fecundity, behavior, growth, development, and physiology in fish can provide regulatory agencies with a better framework to determine potential regulations and fisheries management approaches.

CHAPTER 2: EFFECTS OF AN ENVIRONMENTALLY DERIVED CONTAMINANTS OF

EMERGING CONCERN MIXTURE ON FATHEAD MINNOWS OVER THREE

GENERATIONS

Introduction

2.1.1 Effects of Human Population Growth on Contaminants of Emerging Concern Presence

Since the Industrial Revolution, the human population has grown exponentially due to health and safety improvements (Ferraro & Andreatta, 2017). Advancements in technology, repurposing of chemicals, and increased disposal/use of existing chemicals have resulted in the presence of contaminants of emerging concern (CECs) in our ecosystem. CECs are chemicals seldom monitored or regulated by environmental agencies despite evidence that these compounds may have adverse health effects on humans or wildlife (EPA, 2008). CECs include chemicals such as pharmaceuticals, personal care products, and industrial plasticizers and are uncommonly monitored or regulated in the environment (EPA, 2008; USGS, 2006). These chemicals include many endocrine disruptors, which mimic hormones, altering molecular signal pathway, leading to changes in the endocrine system (EPA, 2008). Many CECs, specifically pharmaceuticals, are discharged through wastewater treatment plants in urbanized areas. Although wastewater treatment plants effectively eliminate, for example analgesics, most pharmaceuticals are not completely removed (often <10%) (Ankley et al., 2007; Collado et al., 2014). The advancements in analytical and toxicological methodology, has improved our ability to determining how commonly used chemicals affect aquatic and terrestrial organisms across the world.

2.1.2 CEC Occurrence in the Aquatic Environment as Complex Mixtures

CECs often find their way into aquatic ecosystems through urban and agricultural runoff. The United States Geological Survey (USGS), US Fish and Wildlife Service, and US Environmental Protection Agency conducted an analysis of surface water samples and bottom sediment samples taken at

260 sites located on 25 tributaries (located in Minnesota, Wisconsin, Illinois, Michigan, Ohio, and New York) around the U.S. Great Lake area from 2010 to 2014 (Elliot et al., in review). The surface-water grab samples were collected in the spring and fall of each year in a 1L amber glass bottle (Elliot et al., in review). The resulting water samples were analyzed for approximately 200 CECs at the USGS National Water Quality Laboratory in Denver, Colorado (Elliot et al., in review). The results of the analysis were then clustered through the Chemical Mixture Software (Scott et al., 2013) using a two-way cluster analysis to determine the co-occurrence, distribution, composition, and possible toxicity of mixtures (Elliot et al., in review). Watersheds and classes of contaminants (anesthetics, antidepressants, beta blockers, organophosphate based flame retardants, pesticides, polycyclic aromatic hydrocarbons, and stimulants) were set as the two factors (Elliot et al., in review). Resulting mixtures were then analyzed for the highest co-occurrence of chemicals in the tributaries. This analysis identified two distinct mixtures: an urban derived mixture and an agricultural derived mixture (Elliot et al, in review).

2.1.3 CEC Effects in Fish

CECs have been found to alter the endocrine system in fish (EPA, 2008). In female *P. promelas*, gonadotrophin-releasing hormone is secreted to the pituitary gland in the hypothalamus, signaling the release of luteinizing and follicle-stimulating hormones (Hadley & Levine, 2006). These hormones facilitate ovarian cells to release androgens and aromatase production, respectively (Hadley & Levine, 2006). Aromatase converts androgens into estrogens, which is responsible for oocyte development and hepatic secretion of vitellogenin (Hadley & Levine, 2006; Jensen et al., 2001). Vitellogenin, an egg-yolk precursor protein, is stored in growing oocytes to serve as a food reserve for developing embryos (Sumpter & Jobling, 1995). Unlike female *P. promelas,* whose plasma vitellogenin concentrations rise during sexual maturation, male *P. promelas* have a silent vitellogenin gene (Sumpter & Jobling, 1995). Exposure to estrogenic CECs activates the vitellogenin gene and results in vitellogenin biosynthesis in

male *P. promelas*, leading to the use of plasma vitellogenin as an indicator of estrogen exposure in male

fish (Panter et al., 1998; Sumpter & Jobling, 1995).

In the case of larval and juvenile fish, exposure to CECs impaired predator avoidance (C-start

response) (McGee et al., 2009), decreased foraging behavior (Brown et al., 1985; Bisei Jr, Bridges, &

Klaine, 2014), delayed visual signals (Wibe et al., 2001), disrupted swimming performance and activity

(Chou et al. 2010), and decreased olfactory responses (Scott et al, 2003; Beyers and Farmer, 2001, Saglio

& Trijasse, 1998).

2.1.4 Mixture Exposure of Fish

In the environment, fish are exposed to a mixture of chemicals from various sources. In these

complex mixtures, there are pharmaceuticals, industrial byproducts, personal care products, and even

industrial chemicals such as anti-icing agents present. A complex mixture is then defined as a mixture

with multiple components in which any estimation of the individual chemicals' toxicities has too much

uncertainty and error to be useful (USEPA, 2000). The individual chemicals can differ in composition

overtime (i.e. one chemical is 50% of the mixture one day and 20% the next month) depending on the

time and conditions the complex mixture is created in the environment (USEPA, 2000). A simple

mixture, as defined by the USEPA (2000), is "a mixture containing two or more identifiable components,

but few enough that the mixture toxicity can be adequately characterized by a combination of the

components' toxicities and the components' interactions" (USEPA, 2000, p. B-2).

In one instance a single chemical exposure to a pharmaceutical was found to increase female

fecundity, but to also decrease fish sociality (Klaminder et al., 2014; Brodin et al., 2013). In another case

where two antagonizing chemicals (17ß-estradiol and nonylphenol) were used in an exposure of

zebrafish, the researchers found the two chemicals behaved additively and non-additively, in reference to

vitellogenin induction, depending on the concentration of the two chemicals used (Lin & Janz, 2006).

Japanese medaka exposed to different mixtures of E2, tamoxifen, and letrozole had adverse effects on

fecundity, hepatic somatic index, and fertility (Sun, Zha, & Wang, 2009). Exposure of E2 and tamoxifen was found to impair fecundity and vitellogenin values more severely (synergistic), while exposure to E2 and letrozole were weakly antagonistic, but E2 effects were too strong for letrozole to completely antagonize (Sun, Zha, & Wang, 2009). Jukosky et al. (2007) exposed adult medaka to environmentally relevant concentrations of 17α-estradiol (EE2), E2, and nonylphenol (simple mixtures) for 14 days. The researchers found lower vitellogenin concentrations in fish exposed to the mixture than those exposed to E2 (Jukosky et al., 2007). They suggested relative estogenicity (additive mixture models) of compounds is useful for predicting some mixture effects (specifically for reproductive), but not for all biological endpoints (Jukosky et al., 2007). Other studies have correlated CEC mixture exposure with intersex in white suckers (Vadja et al., 2008), rainbow darters (Hicks et al., 2017), and smallmouth bass (Blazer et al., 2014). These studies exemplify how difficult it is to determine the biological effects of CECs on fish and whether CECs act synergistically, antagonistically, or additively in a mixture (both simple and complex).

2.1.5 Exposure Length

Some short-term (<21 days) exposures using various fish species, such as zebrafish, Japanese medaka, *P. promelas*, and Chinook salmon, characterizing CEC effects, as single and mixture exposures, have found delayed effects on reproductive events due to changes in sex determination (Cripe et al., 2010, Nash, 2004), egg production (Nash, 2004), and phenotypical changes (Cripe et al., 2010). This delayed effect could lead to a potential mischaracterization of the chronic effects CECs have on fish species (Parrot et al., 2017; Overturf et al., 2015). In multigenerational exposures (direct exposure of an organism over multiple generations, Skinner 2008), the most common adverse outcome is a decrease in fecundity (Cripe et al., 2010; Raimondo et al,. 2009). Kidd et al. (2007), conducted one of the most publicized exposures of *P. promelas* using a synthetic estrogen. The researchers examined a population of *P. promelas* in the Experimental Lakes Area in Ontario, Canada over a course of seven years. At the end of

the three-year exposure, they found the entire *P. promelas* population collapsed due to the feminization of males. It should be noted that the biological effects of CEC exposure should be similar between generations (Parrot et al., 2017). Similar biological effects of CEC exposure, but increased sensitivity was seen by Cripe et al. (2010) when three generations of sheepshead minnows were exposed to the androgen, 17ß-Trenbolone. The authors found increasing adverse impacts on reproduction (cumulative reproduction, daily egg production, and abnormal egg production) for all three generations, finding an interaction effect between treatment and generation. In addition, to reproductive effects, all first-generation fish were phenotypically male (Cripe et al., 2010).

The findings of Cripe et al. (2010) and Kidd et al. (2007) show how exposure of the parental generation ultimately affects subsequent generations, regardless of whether they are exposed or not. These results provide the rationale for testing the hypothesis that fish exposed over subsequent generations experience increased sensitivity to the chemicals (observable effects at subsequent lower doses) due to the exposure of eggs during embryogenesis (Parrot et al., 2017).

2.1.6 Hypotheses

Based on previous research presented above, the goal of this research was to test the hypotheses that, (1) fish from subsequent generations would exhibit the same effects (adverse/therapeutic) when exposed and (2) subsequent exposed generations would be more sensitive, eliciting a more severe biological response when exposed to a complex, urban CEC mixture. To test our hypotheses, we conducted a year-long flow-through exposure experiment with *P. promelas* over three generations in the laboratory.

2.2 Materials and Methods

2.2.1 Exposure Chemicals

Chemical stock solutions were prepared by United States Geological Survey's National Water

Quality Laboratory (Denver, CO) in 100% ethanol (EtOH). Aliquots were stored at 4°C until use.

Chemicals in each mixture included Bisphenol-A (BPA), N,N-Diethyl-meta-toluamide (DEET),

Tributylethly phosphate (TBEP), Methyl-1H-benzotriazole (5-MeBt), Nonylphenols, Galaxolide

(HHCB), Estrone (E1), Sulfamethoxazole, Desvenlafaxine, Fexofenadine, and Metformin. Ethanol

controls were exposed at 100% EtOH equivalent to all treatments (Table 1). The concentrations of each

chemical was determined from surface water samples analyzed in a two-way cluster analysis as described

in Elliot et al. (in review).

Table 1. Concentration (ng/L) of each individual chemical as follows: Bisphenol-A (BPA), *N,N*-Diethyl-3-methylbenzamide (DEET), Tributylethylphosphate (TBEP), 5-methylbenzotriazole (5-MeBt), Nonylphenol, Galaxolide (HHCB), Estrone (E1), Sulfamethoxazole, Desvenlafaxine, Fexofenadine, and Metformin in the mixture for Low (1/10x), Medium (1x; highest environmental concentration), and High (10x) treatments.

Chemical	Low (1/10x) concentration ng/L	Medium (1x) concentration ng/L	High (10x) concentration ng/L.
BPA	300	3,000	30,000
DEET	160	1,600	16,000
TBEP	1,350	13,500	135,000
5-MeBt	668	6,680	66,800
Nonylphenol	371	3,710	37,100
HHCB	218	2,180	21,800
E1	0.69	6.9	69
Sulfamethoxazole	55.9	559	5,590
Desvenlafaxine	58.3	583	5,830
Fexofenadine	100	1,000	10,000
Metformin	121	1,210	12,100

2.2.3 Exposure Organisms

Adult *P. promelas* (6-7 months old, hereafter "F1 generation") were obtained from a laboratory fish rearing facility (Environmental Consulting & Testing, Superior, WI), and were acclimated in approximately prior to exposure. The second generation (hereafter "F2 generation") spawning pairs, were established by collecting eggs from the F1 generation and rearing these fish. Likewise, the third generation (hereafter "F3 generation") was raised from eggs spawned by the F2 generation. Fish were maintained throughout the study at constant water temperature (22°C) and photoperiod (16:8hrs light: dark) in well water from a dedicated in-house well. All fish were fed frozen brine shrimp (*Artemia* spp.) and frozen blood worms (*Glycera* spp.) *ad libitum* twice daily. Larvae were fed freshly hatched brine shrimp *ad libitum* two to three times daily. The fresh brine shrimp were raised in salt water, collected by a separation funnel, and rinsed with well water prior to larval feeding. All fish maintenance and experiments was carried out in accordance with St. Cloud State University's IACUC guidelines (Protocol # 06-09).

2.2.4 Multigenerational Exposure Setup

Breeding groups of *P. promelas* (one mature female and one mature male) were exposed to a low (1/10x), medium (1x; highest measured environmental concentration; see 'Exposure Chemicals'), or high (10x) concentration of the urban CEC mixture, or to an EtOH solvent control. Each treatment consisted of 20 replicates, expect for the EtOH which had 24 replicates for a sample size of 104 total spawning pairs across five treatments. The tanks were distributed across four racks with 28 tanks on each rack. The three mixture treatments were duplicated by grouping 10 tanks of the same treatment to one head tank per treatment. Racks 1-3 grouped eight EtOH control tanks each to triplicate the control treatment (Figure 2). Additional Blank controls were housed on the fourth rack. F1 generation fish were exposed for 60 days beginning 6 months post-hatch. F2 generation fish were exposed for their entire lifecycle, while F3 generation fish were exposed until approximately four months post-hatch (past sexual differentiation;

Figure 2). F2 generation fish were established from the F1 fish. Siblings for the F2 generation were kept in the same tank until seven months post-hatch when fish were distributed into the F2 generation spawning pairs. To minimize chances for inbreeding when establishing the F2 spawning pairs, a matrix of the surviving individuals and their parental origins was created to minimize sibling paring during the F2 spawning pair designation (Figure 3). This was accomplished by taking the total number of siblings and dividing them among the 20 tanks (keeping the lowest amount of siblings per tank). When spawning pairs were established at eight months post-hatch, fish from tanks with no overlapping parental lineages were paired together when possible.
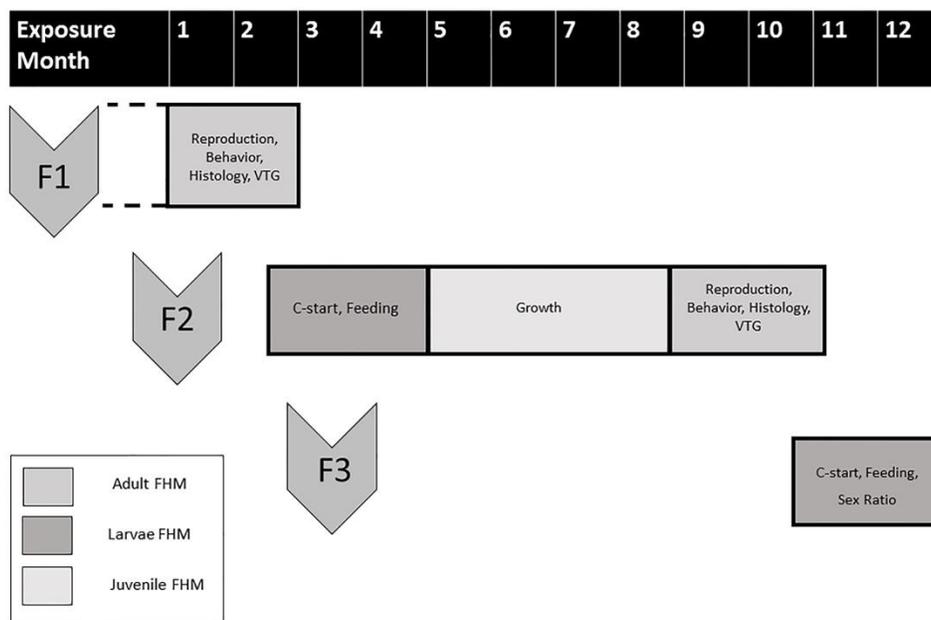
Figure 2. Timeline of *P. promelas* exposure. F1 fish were exposed for two months beginning at six months post-hatch, while F2 fish were exposed for their entire lifetime, and F3 fish were exposed until four months post-hatch. Adults from the F1 and F2 were assessed for biological and behavior endpoints, histology, and vitellogenin (VTG). F2 larvae and juveniles were assessed for c-start behavior, feeding behavior, and Growth. F3 larvae were assessed for c-start behavior, feeding behavior, and sex ratios

EtOH		Old Tank #														SUM of juveniles in new tank
		1	4	25	26	27	30	32	53	54	55	57	59	82	84	
NEW TANK #	11	1			1	2	1		1	1	1	1		1		10
	12	1			1	2	1	1	1	1			1	1		10
	13	1			1	2	1	1	1	1			1	1		10
	14	1		1	1	2	1	1	1				1	1		10
	15	1		1	1	2	1	1	1	1				1		10
	16	1		1	1	2	1	1	1	1				1	1	11
	17	1		1	1	2	1	1	1	1				1	1	11
	18		1	1		2	1	1	1	1			1	1	1	11
	19		1	1	1	1	1	1	1	1			1		1	10
	20			1	1	2		1	1	1	1		1		1	10
SUM of juveniles from old tank		7	2	7	9	19	9	9	10	9	2	1	6	8	5	

Figure 3. An example of the matrix used to redistribute F2 juveniles based on parental lineage

Consistent chemical exposure was ensured by adding a concentrated stock solution containing all CECs in the mixture to the flow of well water into a mixing chamber and then into ten replicate treatment tanks via gravity. The concentrated stock solution was generated every three days by adding a three mL spike of CECs dissolve din absolute EtOH to 10L of well water (St. Cloud State's University Aquatic Toxicology Laboratory dedicated well) in a black tinted glass carboy to decrease the possibility of photolysis. A peroxide cured silicon line (Cole-Parmer, Vernon Hills, IL) was used to pump the solution at a nominal rate of 2.5mL/min with a Masterflex 7523-40 peristaltic pump (Cole-Palmer, Vernon Hills, IL) into a mixing chamber. Well water was fed into a mixing chamber at a rate of 200 mL/min to achieve the final treatment concentrations. The final solution was gravity-fed to the ten replicate tanks (Tygon S3; Pentair, Minneapolis, MN) extended from the bottom of the mixing chamber. Water samples were taken every third exchange by filling a 25-mL amber vial of the final solution. Water samples were frozen until

confirmatory chemical analysis could be performed at the USGS National Water Quality Laboratory (Denver, CO).

2.2.5 Biological Endpoints

A total of 13 biological endpoints were assessed for F1, F2, and F3 generation fish (Table 2).

Table 2. Biological endpoints (Survival, Fecundity, Body Condition Factor [BCF], Hepatic Somatic Index [HSI], Gonadal Somatic Index [GSI], Hematocrit, Glucose, Vitlleogenin [VTG], Histology, C-start, Growth, and Feeding Assay) assessed for each exposure generation (F1-F3) of *P. promelas*.

Biological Endpoints	F1	F2	F3
Survival	Yes	Yes	Yes
Fecundity	Yes	Yes	
BCF	Yes	Yes	
HSI	Yes	Yes	
GSI	Yes	Yes	
SSC	Yes	Yes	
Hematocrit	Yes	Yes	
Glucose	Yes	Yes	
VTG	Yes	Yes	
Histology	Yes	Yes	
C-start		Yes	Yes
Growth		Yes	Yes
Feeding Assay		Yes	Yes

Survival. Survival was calculated as a percentage as follows: Survival= (# of mortalities/# alive) x 100%.

Fecundity. Beginning at Day 21 of the F1 generation exposure, eggs were counted to determine the number of eggs laid (Fecundity= Σ all eggs laid/day/treatment/female). The fecundity for the F2 generation was collected in the same manner once the spawning pairs were established at approximately eight months post-hatch.

Organosomatic Indices. Prior to dissection, individual fish were anesthetized in 0.1% MS-222 (Argent Laboratories, Redmond, WA), measured for length (standard and total length in mm), and weighed (grams). These measurements were used to calculate body condition factor (BCF= body weight/total length^3, Murphy and Willis, 1996). Liver and gonads were dissected and immediately weighed. These weights were used respectively to calculate hepatosomatic index (HSI=liver weight/whole body weight x 100) and gonadosomatic index (GSI=gonad weight/whole body weight x 100).

Secondary Sex Characteristics. Male *P. promelas* sexual characteristics were visually assessed prior to dissection following Parrot et al. (2003) with modifications as follows. Tubercles, dorsal pad, and banded coloration were given a separate rating of 0, 1, 2, or 3 based on prominence (0 being least prominent, 3 being most prominent). The three values were analyzed separately and as a single composite value (0-9) to compare morphological sexual maturity.

Hematocrit. Blood was drawn from the severed caudal vasculature using a 75mm length heparinized capillary tube (ClearCRIT Plastic Capillary Tubes, Separation Technology Inc., Sanford, FL) and sealed (Critoseal, Oxford Labware, St. Louis, MO), centrifuged (HERMLE Z200A, Labnet International Inc., Woodbridge, NJ) for 12 minutes at 5000 x g and 4C, and measured (Spiracrit Micro-Hematocrit Tube Reader, Clay-Adams Inc., New York, NY).

Plasma Vitellogenin Analysis. Blood was drawn from the severed caudal vasculature using a heparinized capillary tube. The heparinized capillary blood tubes were centrifuged at 5000 x g for 12 minutes at 4°C to obtain plasma, which was stored at -80°C prior to ELISA analysis.

Plasma vitellogenin concentrations (μg/mL) were quantified using a competitive antibody-capture ELISA with an eight-point serial dilution standard (4.8 μg/mL to 0.0375 μg/mL) as described in Bartell and Schoenfuss (2012).

Blood Glucose. 1 μL of whole blood taken from a severed caudal vasculature was measured to quantify blood glucose concentration by a TRUEbalance Blood Glucose Monitor (Moore Medical, Farmington, CT) with a detection range of 20 to 600 mg/dL.

Histology. Liver and gonads were dissected from fish (F1 and F2 generation), placed in histological cassettes, and kept in 10% buffered formalin for at least 24-hours. The tissues underwent dehydration, tissue preparation, and paraffin embedding as previously described in (reference).

Liver and gonad samples of ~125mm^3 volume were sectioned at 5 μm (Olympus Cut 4055 Microtome, Olympus America Inc., Center Valley, PA). The tissues were stained using a standard haematoxylin and eosin counter stain (Carson, 1997; Gabe, 1976). Each tissue was randomly and blindly assessed for liver vacuolization and gonadal maturity to eliminate observational bias.

Liver vacuolization were graded on a scale of 1 to 4 at 40x magnification. A grade of 1 indicated there were no vacuoles visible; a grade of 2 indicated less than 25% of the tissue had vacuoles; a grade of 3 indicated 25-50% of the tissue had vacuoles; and a grade of 4 indicated majority of the tissue consisted of vacuoles (Figure 4).

Testes were assessed based on the various stages of sperm cell development. A percentage of spermatogonia, spermatocytes, spermatids, and spermatozoa were assed for each tissue (Figure 4). The overall maturity of the sample was calculated as a Gonad maturity rating = $((\%_{spermatogonia})+(\%_{spermatocytes}$ x $2)+(\%_{spermatids}$ x $3)+(\%_{spermatozoa}$ x $4))/100$.
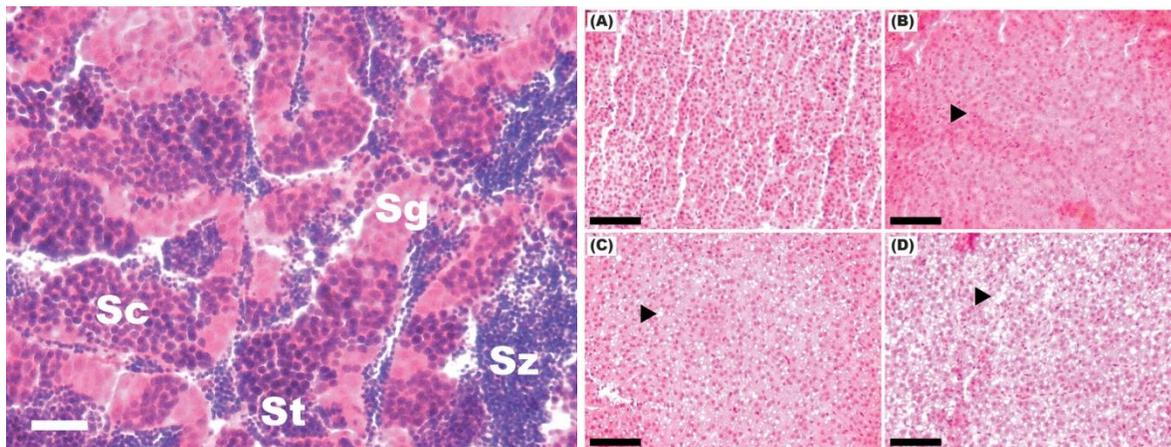
Figure 4. Histology examples of P. promelas liver vacuolization (left) and testes with stage 1-4 (A-D) of sperm cell development (right).

Larval c-starts and Feeding Assay. C-start performance of F2 and F3 generation larvae were measured using a trigger-activated system as described in Painter et al. (2009). The resulting video was analyzed using ImageJ for response latency (BL/ms), body length (mm), velocity (ms), total escape response (BL/ms), and escape angle (°C).

In addition to the C-start performance assay, a feeding assay was conducted. Larvae were starved for 12-18 hours before testing. Individuals were placed into a well on a clear, plastic, six well plate and

allowed to acclimate overnight. The following morning, 13-17 newly hatched brine shrimp were counted and added to each individual well. The larvae were allowed to eat for one minute before being pipetted out of the well. Brine shrimp were then immobilized with formalin and counted to determine the number remaining. The difference between original brine shrimp count and count after foraging was assumed to be consumed by the larval *P. promelas*.

Growth. Growth for the F2 and F3 generation was assessed at 21-days post-hatch using recordings obtained during the c-start assay and analyzed using ImageJ.

Statistical Analysis. Assumption of normality for all data sets were tested with a Sharpio-Wilks test before any additional analysis. An $\alpha=0.05$ was set for all comparisons. Outliers were determined by analyzing Cook's Distance. All ratios (BCF, GSI, HSI) were arcsine transformed for analysis. Others, such as vitellogenin and hematocrit, were log-transformed prior to analysis. All variables were tested with a generalized linear model (GLM) for effects of treatment, sex, generation, and their interactions. When the GLM assessed no significant differences of the variables, data were pooled together. Differences in Fecundity were analyzed with a repeat measures ANOVA with a Holm-Sedak post-test when applicable. The variables BCF, GSI, HSI, hematocrit, vitellogenin, glucose, liver vacuolization, GPA, and sum of sex characteristics (tubercles, dorsal pad, color) were analyzed using a one-way analysis of variance (ANOVA) to test effects of treatment. Tukey's post-test was used for all pairwise comparisons. Larval feeding assay data was analyzed using a GLM with a negative Poission log link to account for the possibilities of zeroes in number of brine shrimp eaten.

2.3 Results

2.3.1 Adult Exposure

Survival. Survival was greater than 90% in all generations (Figure 5). High treatment P. promelas had the lowest survival at 90%, followed by EtOH (93.75%), Medium (97.50%), and Low (100%). F2 adult P. promelas had 100% survival in all treatments.
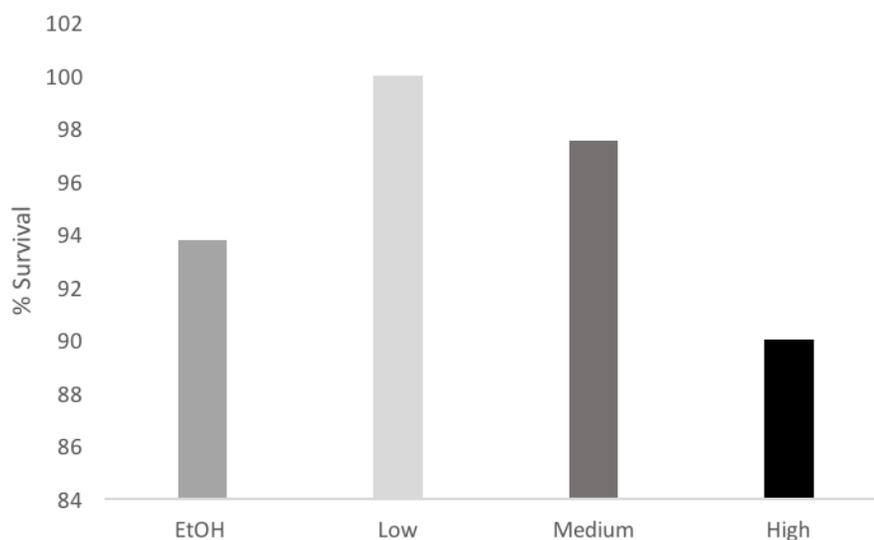
Figure 5. Percent Survival of F1 P. promelas exposed to EtOH, Low, Medium, and High treatments over 60 days.

       Fecundity. In both F1 and F2, the mean number of eggs spawned per female per day was lower at high concentrations, than at low concentrations (Figure 6). Overall, F2 generation female P. promelas had a lower number of eggs produced over the course of the exposure (Table 3 and 11). F1 Low Treatment females had higher cumulative mean number of eggs laid per day than EtOH, Medium or High Treatment, while Medium Treatment F1 generation females had significantly higher fecundity than EtOH or High Treatment fish. ($p<0.05$; Table 13) In the F2 generation, all treatments were significantly different from each other ($p<0.05$; Table 13).

Table 3. Repeat Measures ANOVA results for F1 and F2 Fecundity data.

Generation	Effect	F	df	P
F1	Treatment	34	3	<0.0001
F2	Treatment	32	3	<0.0001
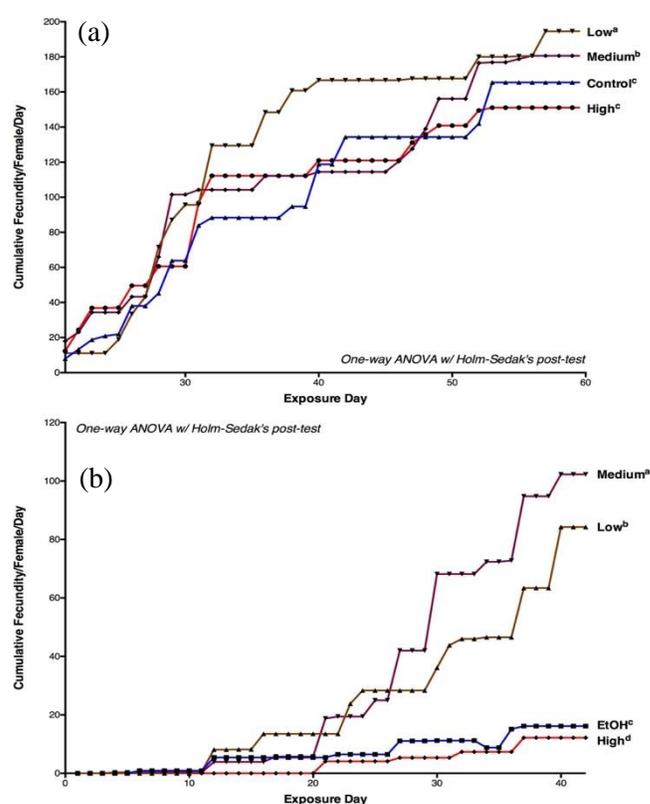
Figure 6. F1 (a) and F2 (b) Fecundity, calculated as cumulative mean number of eggs laid per female

P. promelas per day by treatment (EtOH, Low, Medium, High) beginning after 22 days of exposure

until day 60 for F1 and when spawning pair were created at eight months post-hatch for F1 and when

BCF, HSI, GSI. Based on the GLM ran for BCF, there is a sex, generation, and interaction (Sex

x Generation) effect on BCF given the other variables and their respective interactions (Table 4), but no

treatment effect. No outliers were found in the data. Overall, F1 females had a higher BCF than F1

males, F2 males, and F2 females (Fig. 7). When looking at differences between treatments, F2 females

exposed to EtOH had smaller BCF values than F1 females exposed to Low and High treatments ($p<0.008$

and $p<0.001$; see Figure 7). F2 females exposed to the Low treatment also had smaller BCF values

compared to F1 Low and High treatment exposed females ($p<0.015$ and $p<0.001$; Fig. 7). F2 females

exposed to Medium and High treatments had lower BCF values than F1 females exposed to the High

treatment ($p<0.005$ and $p=0.001$; Fig. 7). There were no differences between males of generations or

treatment.

Table 4. Results of GLM examining the effects of treatment (EtOH Control, Low, Medium, High), sex,

and Generation (F1 and F2) on the Body Condition Factor (BCF) of adult *P. promelas*. Significant effects

are given in bold ($\alpha < 0.050$).

Effect	F	df	P
Treatment	2.0474	3	0.1073
Sex	13.4131	3	**0.0003***
Generation	13.9514	1	**0.0002***
Treatment *x* Sex	0.4336	3	0.7291
Treatment *x* Generation	0.5156	3	0.6718
Sex *x* Generation	35.5879	3	**<0.0001***
Treatment *x* Sex *x* Generation	0.8925	1	0.4453
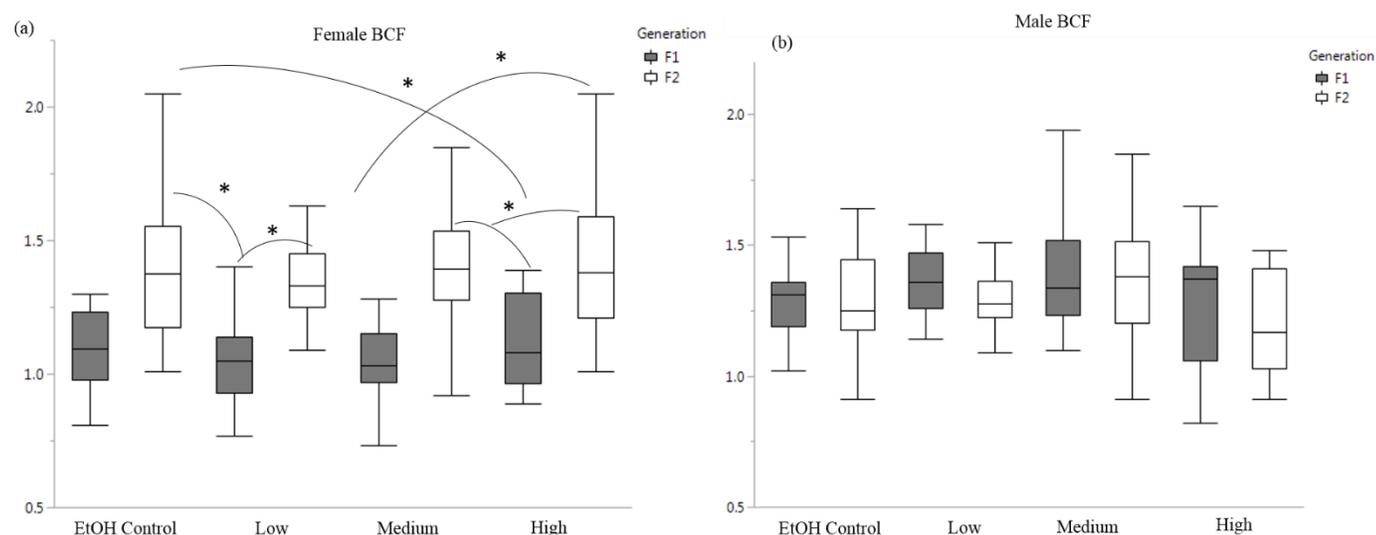
Figure 7. Box and Whisker plots of Body Condition Factor of F1 and F2 female (a) and male (b) *P. promelas* showing mean, range, and 95% confidence intervals. F1 generation are indicated by the shaded boxes. * denote significant differences at α=0.05.

Based on the GLM ran for HSI, there is a sex, generation, and interaction (Sex *x* Generation) effect on HSI given the other variables and their respective interactions, with no outliers (Table 5). Overall, F1 females had higher HSI values than F1 males, F2 females, and F2 males, but there was no treatment effect (Fig. 8). There were no differences in F1 and F2 females or males in treatment.

Table 5. Results of GLM examining the effects of treatment (EtOH Control, Low, Medium, High), sex, and Generation (F1 and F2) on the Hepatic Somatic Index (HSI) of adult *P. promelas*. Significant effects are given in bold (α < 0.050).

Effect	F	*df*	*P*
Treatment	0.2583	3	0.8554
Sex	22.5097	1	**<0.0001***
Generation	12.0315	1	**0.0006***
Treatment *x* Sex	1.8086	3	0.1456
Treatment *x* Generation	0.3187	3	0.8119
Sex *x* Generation	4.3497	1	**0.0379***
Treatment *x* Sex *x* Generation	0.8374	3	0.4742
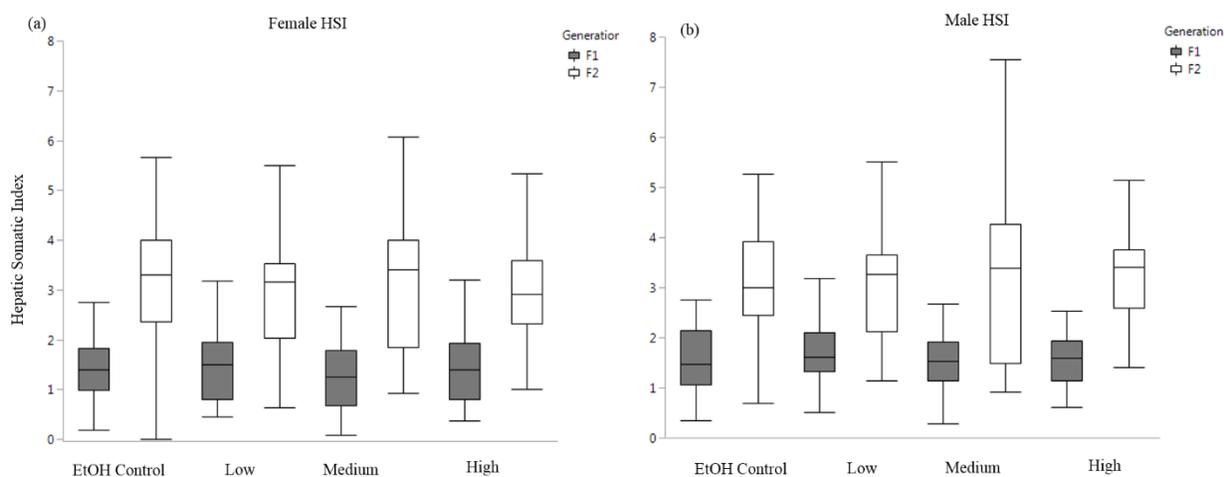
Figure 8. Box and Whisker plots of Hepatic Somatic Index of F1 and F2 female (a) and male (b) *P. promelas* showing mean, range, and 95% confidence intervals. F1 generation are indicated by the shaded boxes.

Based on the GLM ran for GSI, there is no sex, generation, or generation effect or outliers (Table 6; Fig 9).

Table 6. Results of GLM examining the effects of treatment (EtOH Control, Low, Medium, High), sex, and Generation (F1 and F2) on the Gonadal Somatic Index (GSI) of adult *P. promelas*.

| Effect	F	*df*	*P*
Treatment	0.6311	3	0.5955
Sex	2.8081	1	0.0949
Generation	0.0062	3	0.9373
Treatment *x* Sex	0.2393	3	0.8689
Treatment *x* Generation	0.8917	3	0.4459
Sex *x* Generation	0.0596	1	0.8073
Treatment *x* Sex *x* Generation	0.4775	3	0.6982
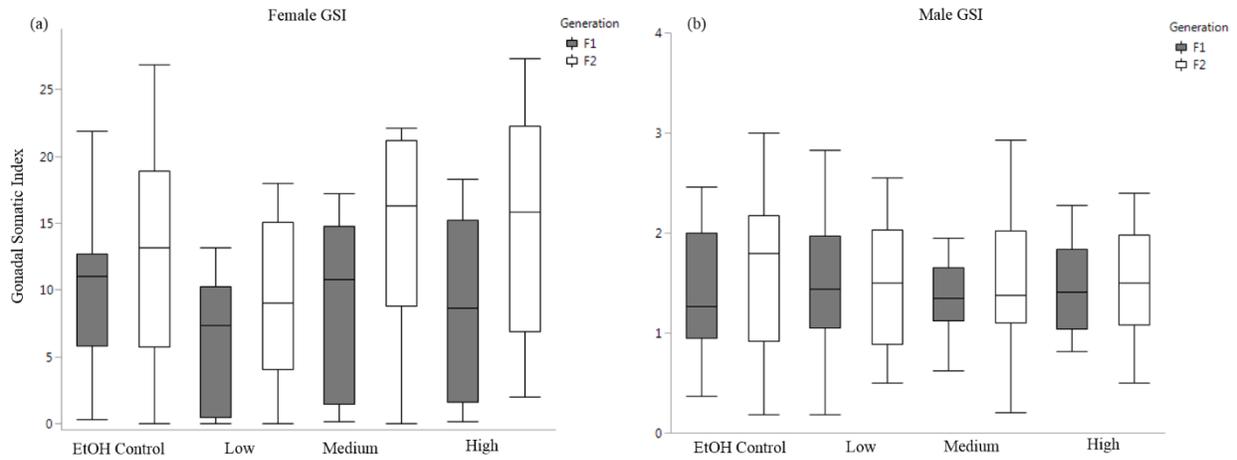
Figure 9. Box and Whisker plots of Gonadal Somatic Index of F1 and F2 female (a) and male (b) *P. promelas* showing mean, range, and 95% confidence intervals. F1 generation are indicated by the shaded boxes.

Secondary Sex Characteristics The GLM showed there was a treatment and generational effect on the Sum of SSC of adult male *P. promelas* (no outliers, Table 7). F1 Males exposed to EtOH control were visually more dominant than F2 males exposed to the High treatment (p=0.0151; Fig 10). Low treatment F1 males were also more phenotypically dominant than F2 EtOH, Medium, and High treatment males (p=0.0111, p=0.0056, p=0.0007; Fig. 10).

Table 7. Results of GLM examining the effects of treatment (EtOH Control, Low, Medium, High) and Generation (F1 and F2) on the Sum of Secondary Sex Characteristics (Sum of SSC) of adult male *P. promelas*. Significant effects are given in bold ($\alpha < 0.050$).

Effect	F	df	P
Treatment	3.6954	3	**<0.0001***
Generation	20.9366	1	**0.0133***
Treatment *x* Generation	0.2819	3	0.9924
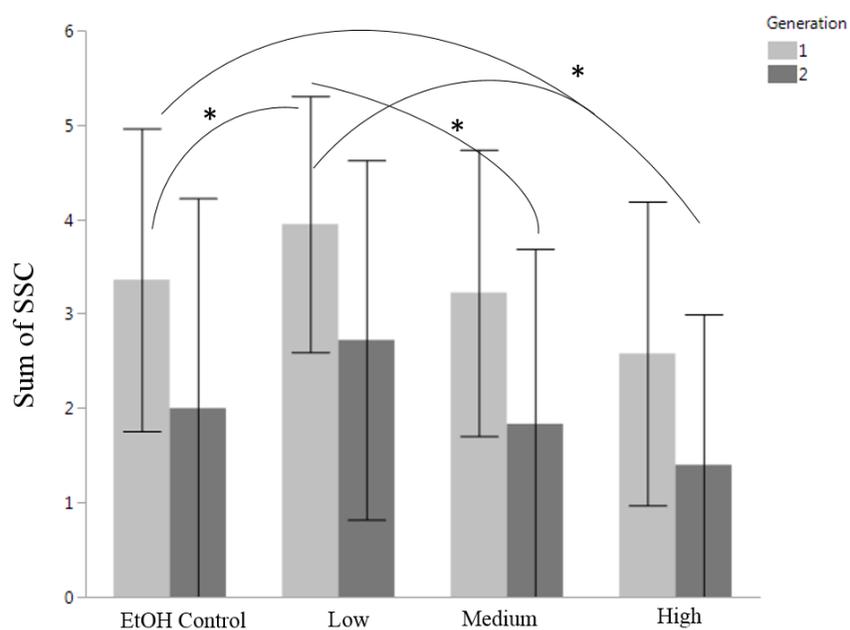
Figure 10. Bar graph of Sum of Secondary Sex Characteristics for male *P. promelas* exposed to EtOH, Low, Medium, and High Treatments with standard deviation error bars. * denote significant differences at $\alpha=0.05$.

Hematocrit. Based on the GLM ran for hematocrit, there is a sex and interaction (Sex x Generation) effect on hematocrit given the other variables and their respective interactions (no outliers, Table 8). Despite those significances, there was no difference between F1 and F2 females of different treatments or with F1 and F2 males (Fig 11).

Table 8. Results of GLM examining the effects of treatment (EtOH Control, Low, Medium, High), sex, and Generation (F1 and F2) on the hematocrit values of adult *P. promelas*. Significant effects are given in bold ($\alpha < 0.050$).

Effect	F	*df*	*P*
Treatment	1.9549	3	0.1211
Sex	24.9827	1	**<.0001***
Generation	0.2306	3	0.6315
Treatment *x* Sex	1.8616	3	0.1363
Treatment *x* Generation	0.9622	3	0.4110
Sex *x* Generation	4.8345	1	**0.0287***
Treatment *x* Sex *x* Generation	0.9167	3	0.4332
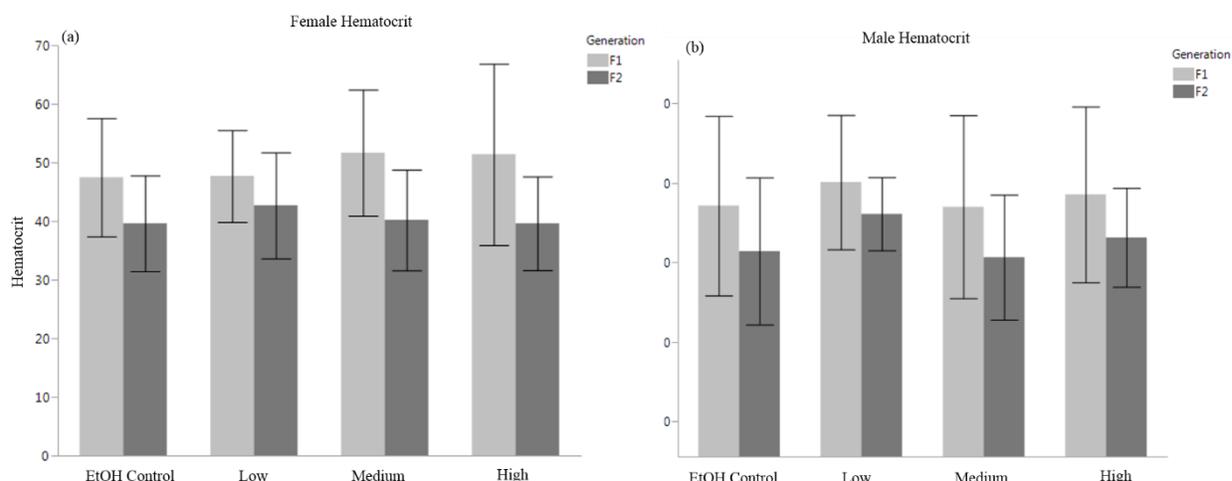
Figure 11. Bar graph of hematocrit (% of plasma to red blood cells) for F1 and F2 female (a) and male (b) *P. promelas* exposed to EtOH, Low, Medium, and High Treatments with standard deviation error bars.

Glucose. Based on the GLM ran for Glucose, there is a sex, generation, and interactions (Treatment x Generations; Treatment x Sex and Sex x Generation) effect on BCF given the other variables and their respective interactions (no outliers, Table 9). Overall, females had higher glucose values than males ($p < 0.050$; see Tables 13 and 14). F1 females exposed to EtOH and Low treatments had lower glucose values than F2 females exposed to EtOH ($p = 0.024$ and $p = 0.017$; Fig 12). There were no differences for F1 and F2 males (Fig 12).

Table 9. Results of GLM examining the effects of treatment (EtOH Control, Low, Medium, High), sex, and Generation (F1 and F2) on the glucose values of adult *P. promelas*. Significant effects are given in bold (α < 0.050).

Effect	F	*df*	*P*
Treatment	1.5532	3	0.2012
Sex	66.5860	1	**<0.0001***
Generation	11.4708	3	**0.0008***
Treatment *x* Sex	2.8791	3	**0.0365***
Treatment *x* Generation	2.8113	3	**0.0399***
Sex *x* Generation	0.3967	1	0.5293
Treatment *x* Sex *x* Generation	2.7362	3	**0.0440***
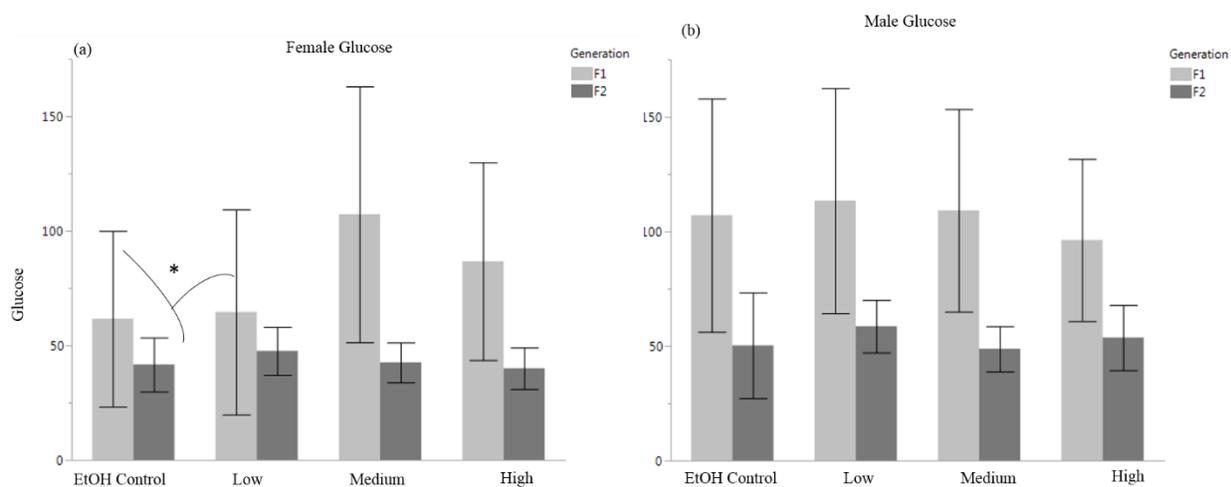
Figure 12. Bar graph of glucose (mg/dL) for F1 and F2 female (a) and male (b) P. promelas exposed to EtOH, Low, Medium, and High Treatments with standard deviation error bars. * denote significant differences at α=0.05.

Vitellogenin. Based on the GLM ran for vitellogenin, there is a treatment, sex, generation, and interactions (Treatment x Sex; Sex x Generation) effect on vitellogenin given the other variables and their respective interactions (Table 10). F1 females exposed to the Medium and High treatments had lower vitellogenin concentrations than F2 females exposed to the High treatment (p=0.045 and p=0.046; Tables 15 and 16). F1 males exposed to EtOH and Low treatments had lower vitellogenin values than F1 males exposed to the High treatment (p=0.0001 and p=0.005; Fig 13). F1 males exposed to the Medium and High treatments had higher vitellogenin values than F2 males exposed to the EtOH and Low treatments (p=0.007, p=0.025, p<0.0001, and p<0.0001; Fig 13).

Table 10. Results of GLM examining the effects of treatment (EtOH Control, Low, Medium, High), sex, and Generation (F1 and F2) on the Vitellogenin of adult *P. promelas*. Significant effects are given in bold (α < 0.050).

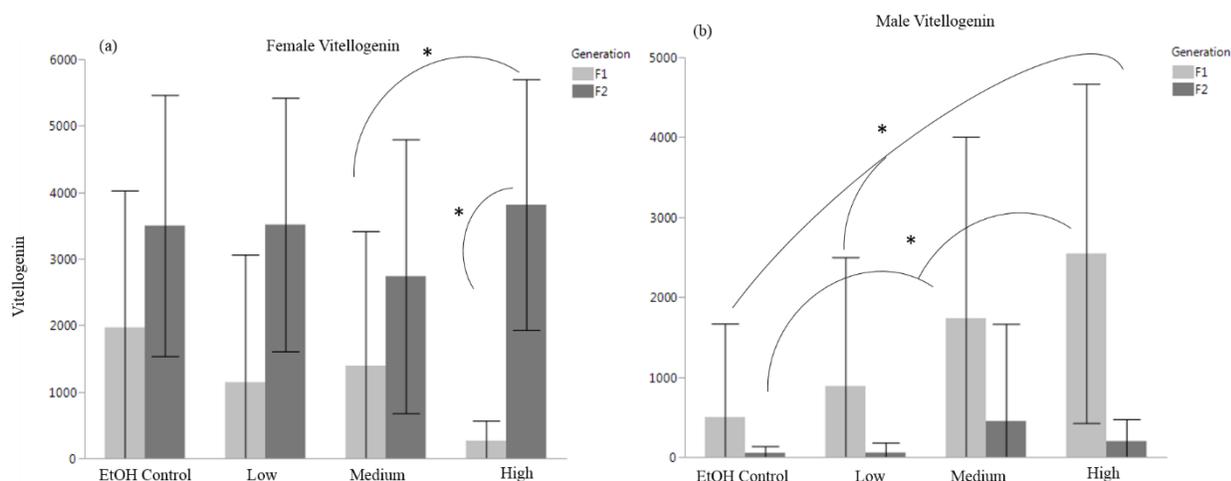| Effect	F	*df*	*P*
Treatment	2.7610	3	**0.0433***
Sex	50.4850	1	**<0.0001***
Generation	4.6718	1	**0.0319***
Treatment *x* Sex	7.3383	3	**0.0001***
Treatment *x* Generation	0.0454	3	0.9871
Sex *x* Generation	43.9956	1	**<0.0001***
Treatment *x* Sex *x* Generation	2.5532	3	0.0567

Figure 13. Bar graph of Vitellogenin (ug/mL) for F1 and F2 female (a) and male (b) P. promelas exposed

to EtOH, Low, Medium, and High Treatments with standard deviation error bars. * denote significant

differences at α=0.05.

Liver Vacuolization. Histology Sex, Generation, and the interaction are all significant effects in

the model (no outliers, Table 11). F1 EtOH females had lower liver vacuolization scores than High and

Low treatment F2 fish (p=0.013 and p=0.0031; Fig 14). F1 females exposed to Low treatment had lower

liver vacuolization than F2 Low treatment females (p=0.0064; Fig 14). F1 and F2 females exposed to

Low treatment had lower and higher liver vacuolization score than F1 and F2 High treatment females,

respectively (p=0.0027, p=0.0062). Likewise, F1 Medium treatment females had lower liver

vacuolization than F2 Low and High treatment fish (p=0.0063, p= 0.0027; Fig 14). In Males, F1 High

treatment males had higher liver vacuolization scores than F2 Low, Medium, and High treatment fish

(p=0.0032, p=0.0027, p=0.0462; Fig 14).

Table 11. Results of GLM examining the effects of treatment (EtOH Control, Low, Medium, High), sex, and Generation (F1 and F2) on the Liver Vacuolization of adult P. promelas. Significant effects are given in bold ($\alpha < 0.050$).

Effect	F	df	P
Treatment	0.5886	3	0.6231
Histology Sex	77.1038	1	**<0.0001***
Generation	69.7632	1	**<0.0001***
Treatment x Histology Sex	0.7755	3	0.5089
Treatment x Generation	2.4827	3	0.0620
Histology Sex x Generation	4.3198	1	**0.0389***
Treatment x Histology Sex x Generation	0.5462	3	0.6512
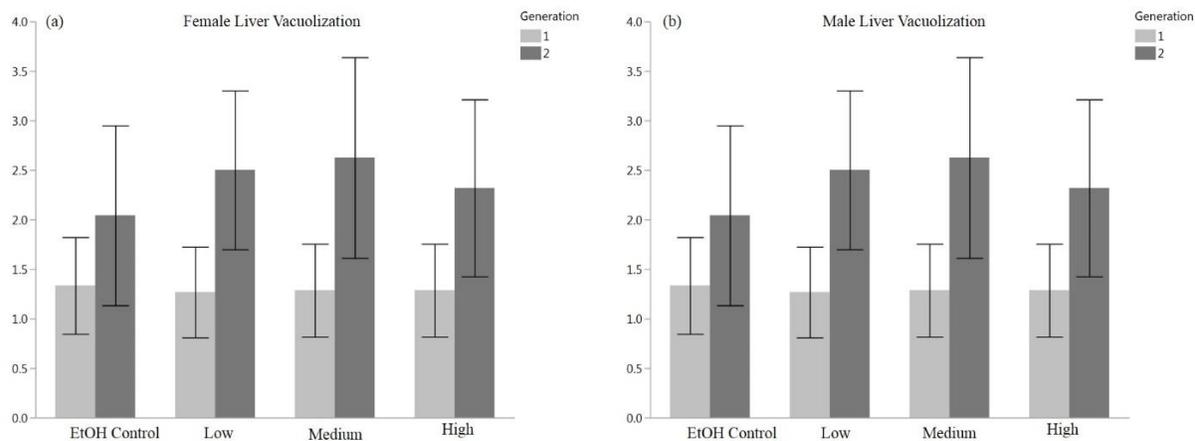
Figure 14. Bar graph of Liver Vacuolization for F1 and F2 female (a) and male (b) *P. promelas* exposed to EtOH, Low, Medium, and High Treatments with standard deviation error bars.

GPA. There was a significant effect of histology confirmed sex on the GPA of F1 and F2 generation *P. promelas* (no outliers, Table 12). However, there were no significant effects of treatment, generation, and the interactions of treatment, sex, and generation.

Table 12. Results of GLM examining the effects of treatment (EtOH Control, Low, Medium, High), sex, and Generation (F1 and F2) on the GPA (Gonadal Maturity) of adult P. promelas. Significant effects are given in bold ($\alpha < 0.050$).

| Effect	F	*df*	*P*
Treatment	2.4703	3	0.0630
Histology Sex	11.3872	1	**0.0009***
Generation	1.0617	1	0.3041
Treatment *x* Histology Sex	0.7406	3	0.5290
Treatment *x* Generation	1.2796	3	0.2825
Histology Sex *x* Generation	1.4023	1	0.2377
Treatment *x* Histology Sex *x* Generation	0.5722	3	0.6339
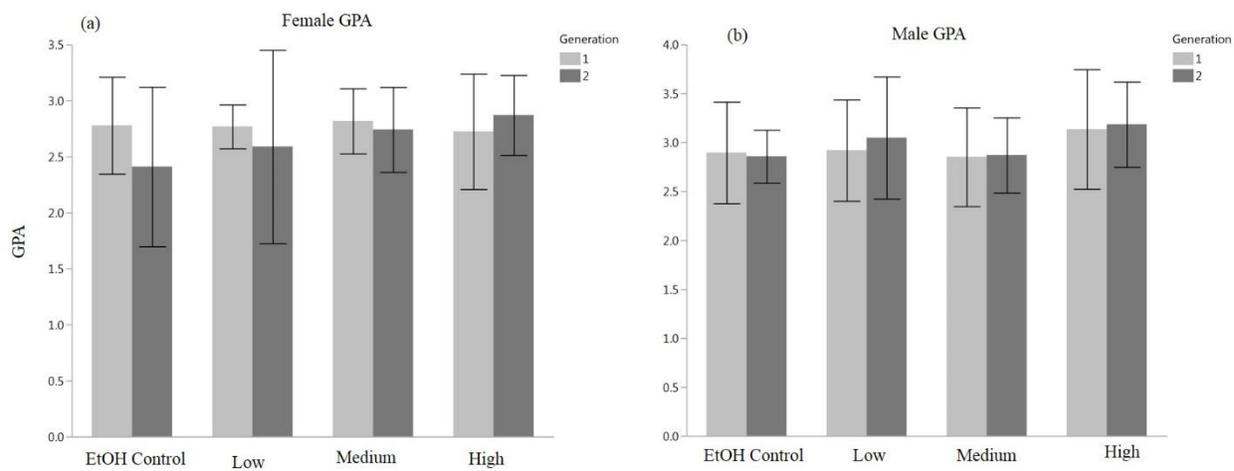
Figure 15. Bar graph of GPA for F1 and F2 female (a) and male (b) P. promelas exposed to EtOH, Low, Medium, and High Treatments with standard deviation error bars.

Table 13. Mean and standard deviation of the following biological indices for Generation 1 (F1) and Generation 2 (F2) adult female *P. promelas* exposed to EtOH Control, Low (1/10x), Medium (1x), and High (10x) treatments: Fecundity, Body Condition Factor (BCF), Gonadal Somatic Index (GSI), and Hepatic Somatic Index (HSI).

Biological Endpoints	F1 – Mature Female *P. promelas* Mean ± std. dev. (n)				F2- Mature Female *P. promelas* Mean ± std. dev. (n)			
	EtOH Control	Low	Medium	High	EtoH Control	Low	Medium	High
Fecundity	103±50	131±60	114±49	107±42	7.1±5.4	26±26	33±37	3.8±4.4
	(39)	(39)	(39)	(39)	(42)	(42)	(42)	(42)
BCF*	1.09±0.14	1.06±0.16	1.05±0.13	1.12±0.18	1.40±0.31	1.36±0.18	1.40±0.25	1.41±0.25
	(18)	(19)	(19)	(20)	(27)	(22)	(22)	(21)
HSI^	1.27±0.63	2.05±2.94	0.99±0.54	1.35±0.88	5.48±6.95	6.03±15.82	4.11±4.87	3.12±1.70
	(18)	(19)	(19)	(20)	(25)	(22)	(20)	(20)
GSI'	13.33±17.38	5.71±4.85	9.14±6.25	8.62±6.76	20.95±28.09	9.35±5.97	14.50±7.27	14.86±8.28
	(18)	(19)	(19)	(20)	(22)	(20)	(17)	(16)

*BCF = body weight/total length^3 ^HSI= liver weight/whole body weight x 100 'GSI= gonad weight/whole body weight x 100

Table 14. Mean and standard deviation of the following biological indices for Generation 1 (F1) and Generation 2 (F2) adult male *P. promelas*

exposed to EtOH Control, Low (1/10x), Medium (1x), and High (10x) treatments: Body Condition Factor (BCF), Gonadal Somatic Index (GSI),

Hepatic Somatic Index (HSI), and the Sum of Secondary Sex Characteristics (Sum of SSC).

Biological Endpoints	F1 – Mature Male *P. promelas* Mean ± std. dev. (n)				F2- Mature Male *P. promelas* Mean ± std. dev. (n)			
	EtOH Control	Low	Medium	High	EtoH Control	Low	Medium	High
BCF*	1.29±0.14	1.36±0.13	1.41±0.26	1.28±0.21	1.31±0.36	1.28±0.14	1.35±0.22	1.26±0.28
	(25)	(20)	(18)	(19)	(20)	(18)	(18)	(15)
HSI^	1.54±0.66	1.69±0.60	1.53±0.69	1.62±0.65	3.02±1.16	3.19±1.23	3.27±1.83	3.20±0.97
	(25)	(20)	(18)	(19)	(19)	(18)	(18)	(15)
GSI'	2.02±3.14	1.49±0.67	1.37±0.46	1.54±0.64	1.79±1.11	2.66±5.12	1.54±0.73	1.54±0.57
	(25)	(20)	(18)	(19)	(16)	(18)	(18)	(14)
Sum of SSC	3.36±1.60	3.95±1.36	3.22±1.52	2.58±1.61	2.00±2.22	2.72±1.90	1.83±1.86	1.4±1.59

*BCF = body weight/total length^3 ^HSI= liver weight/whole body weight x 100 'GSI= gonad weight/whole body weight x 10

Table 15. Mean and standard deviation of the following biological indices for Generation 1 (F1) and Generation 2 (F2) adult female *P. promelas* exposed to EtOH Control, Low (1/10x), Medium (1x), and High (10x) treatments: Hematocrit (% of plasma to red blood cells), Glucose (mg/dL), Vitellogenin (VTG), Gonad maturity (GPA), and liver vacuolization.

Biological Endpoints	F1- Mature Female *P. promelas* Mean ± std. dev. (n)				F2- Mature Female *P. promelas* Mean ± std. dev. (n)			
	EtOH Control	Low	Medium	High	EtOH Control	Low	Medium	High
Hematocrit (% of plasma to red blood cells)	47.47±10.09 (15)	47.69±7.85 (16)	51.68±10.75 (19)	45.94±11.32 (18)	39.63±8.17 (24)	42.68±8.60 (22)	40.18±8.60 (22)	39.61±8.00 (18)
Glucose (mg/dL)	107.13±50.89 (24)	113.48±49.10 (19)	109.19±44.22 (16)	96.26±35.36 (19)	51.30±23.22 (20)	58.61±11.48 (18)	48.78±9.83 (18)	53.73±14.23 (15)
VTG (ug/mL)	1968.82± 2057.26 (10)	1144.41± 1917.23 (8)	1393.09± 2023.53 (13)	265.12± 302.44 (4)	3498.23± 1963.26 (17)	3514.59± 1903.84 (17)	2736.72± 2059.13 (14)	3814.95± 1884.41 (15)

GPA	2.88±0.53	2.85±0.50	3.13±0.61	2.98±0.55	2.86±0.27	2.86±0.39	3.21±0.44	3.05±0.62
	(15)	(10)	(12)	(9)	(17)	(18)	(13)	(13)
Liver Vacuolization	1.23±0.44	1.2±0.42	1.25±0.45	1.13±0.35	2.00±0.94	2.44±1.04	2.07±0.73	2.54±0.8
	(13)	(10)	(12)	(8)	(19)	(18)	(14)	(13)

Table 16. Mean and standard deviation of the following biological indices for Generation 1 (F1) and Generation 2 (F2) adult male *P. promelas* exposed to EtOH Control, Low (1/10x), Medium (1x), and High (10x) treatments: Hematocrit (% of plasma to red blood cells), Glucose (mg/dL), Vitellogenin (VTG), Gonad maturity (GPA), and liver vacuolization.

Biological Endpoints	F1- Mature Male *P. promelas* Mean ± std. dev. (n)				F2- Mature Male *P. promelas* Mean ± std. dev. (n)			
	EtOH Control	Low	Medium	High	EtOH Control	Low	Medium	High
Hematocrit (% of plasma to red blood cells)	47.14±11.30 (21)	50.11±8.47 (19)	47.00±11.53 (16)	48.56±11.08 (18)	41.42±9.26 (19)	46.11±4.60 (18)	40.65±7.88 (17)	43.14±6.24 (14)
Glucose (mg/dL)	61.62±38.37 (13)	64.58±44.76 (12)	107.18±55.80 (11)	88.692±43.11 (13)	42.03±11.78 (27)	47.57±10.47 (21)	42.55±8.69 (22)	40.05±9.03 (20)
VTG (ug/mL)	500.03± 1169.23 (12)	887.10± 1612.04 (16)	1736.96± 2270.41 (14)	2547.78± 2122.27 (16)	54.01± 82.72 (18)	54.75± 124.07 (15)	452.04± 1213.41 (15)	197.80± 274.18 (10)

GPA	2.75±0.44	2.82±0.29	2.72±0.52	2.78±0.20	2.41±0.71	2.71±0.38	2.87±0.37	2.59±0.86
	(12)	(13)	(18)	(13)	(17)	(14)	(15)	(8)
Liver Vacuolization	2.67±1.07	2.64±0.93	2.29±1.05	1.85±0.90	2.84±0.90	3.33±0.90	3.31±0.87	3.13±0.35
	(12)	(14)	(17)	(13)	(19)	(15)	(16)	(8)

2.3.2 Larval Exposure

C-starts. Based on the GLM, there was a generational effect, but no interaction effects (Table 17). At 21 days post-hatch, F3 larvae took greater time to respond to the vibrational stimulus, had a slower escape velocity, and lower escape response than F2 larvae (p=0.012, p<0.001, p=0.002; Fig 16). There were no significant differences in escape angle.

Table 17. Results of GLM examining the effects of treatment (EtOH Control, Low, Medium, High) and Generation (F2 and F3) on latency, escape velocity, total escape response, and escape angle of larval *P. promelas*. Significant effects are given in bold ($\alpha < 0.050$).

Effect	Latency			Escape Velocity			Total escape Response			Escape Angle		
	F	*df*	*P*	F	*df*	*P*	F	*df*	*P*	F	*df*	*P*
Treatment	1.5581	3	0.2007	0.2792	3	0.8403	0.2901	3	0.8325	1.0184	3	0.3857
Generation	6.3665	1	**0.0124***	26.4617	1	**<.0001***	10.6432	1	**0.0013***	0.6523	1	0.4203
Treatment *x* Generation	0.5046	3	0.6795	0.4079	3	0.7475	0.3017	3	0.8241	1.4647	3	0.2256
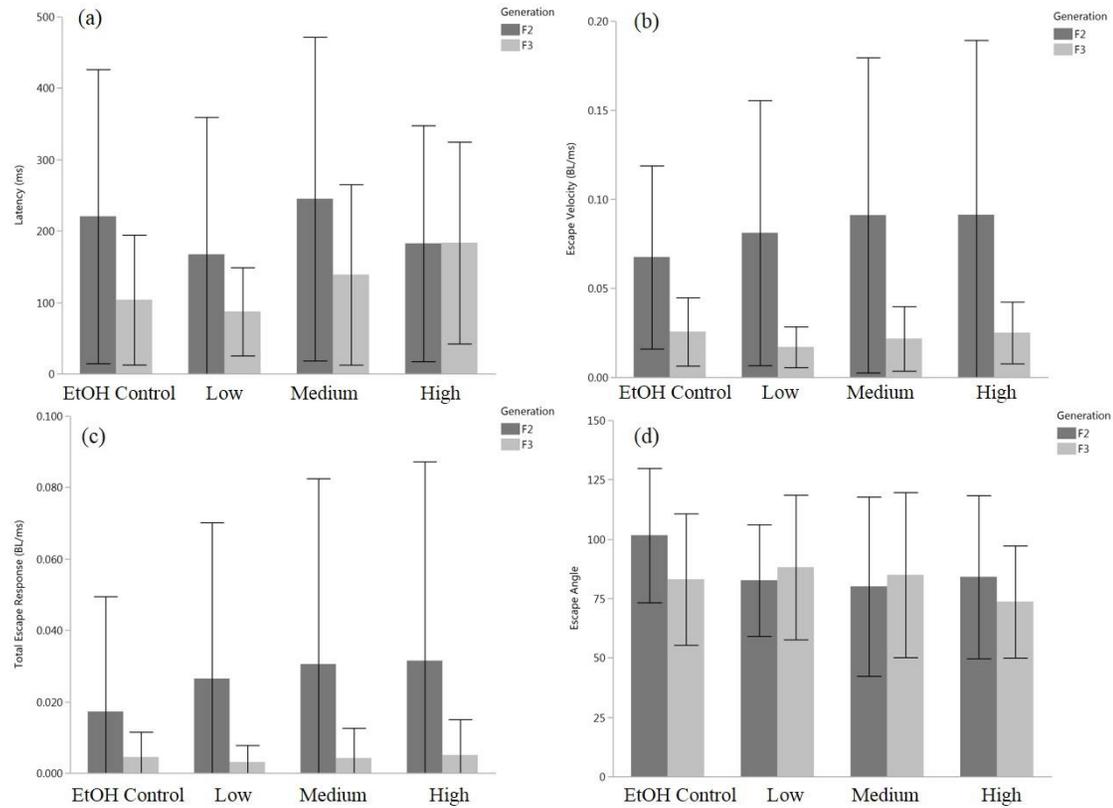
Figure 16. Bar Graph of Latency (ms, a), Escape Velocity (BL/MS, b), Total Escape Response (BL/ms, c), and Escape Angle (degrees, d) at 21 days post-hatch for F2 and F3 *P. promelas* with standard deviation error bars for larvae exposed to EtOH, Low, Medium, and High Treatments.

Table 18. Larval C-start Endpoints of Latency (ms), Velocity (Body Length/ms), Total Escape Response (Body Length/ms), and Escape Angle (degrees), Growth (mm), and Feeding (% consumed) results for F2 and F3 larvae 21-days post-hatch.

Biological Endpoint	F2 Mean ± std. dev. (n)				F3 Mean ± std. dev. (n)			
	EtOH Control	Low	Medium	High	EtOH Control	Low	Medium	High
C-start Latency (ms)	220.27±205.90 (26)	166.97±192.33 (31)	244.82±226.63 (38)	182.40±165.19 (40)	103.39±90.90 (18)	87.00±61.64 (23)	138.72±126.338 (36)	183.25±141.31 (4)
C-start Velocity (BL/ms)	0.07±0.05 (26)	0.08±0.07 (31)	0.09±0.09 (38)	0.09±0.10 (40)	0.03±0.02 (18)	0.02±0.01 (23)	0.02±0.02 (36)	0.03±0.02 (4)
C-start Total Escape	0.02±0.03 (26)	0.03±0.04 (31)	0.03±0.05 (38)	0.03±0.06 (40)	0.004±0.007 (18)	0.003±0.005 (23)	0.004±0.008 (36)	0.005±0.010 (4)

Response (BL/ms)							
C-start Escape Angle (degrees)	101.55±28.28 (23)	82.60±23.50 (26)	80.03±37.76 (30)	84.03±34.34 (33)	83.03±27.66 (18)	88.09±30.45 (23)	84.89±34.74 (36)	73.60±23.62 (4)
Growth (mm)	8.41±1.35 (26)	8.11±1.49 (31)	7.61±1.39 (38)	7.21±1.93 (40)	12.89±2.56 (19)	12.64±2.06 (23)	9.79±2.70 (37)	14.75±3.72 (4)
Feeding Assay (% consumed)	7.39±1.49 (28)	6.21±1.37 (24)	6.15±1.16 (33)	3.40±0.612 (40)	8.32±2.02 (19)	11.95±2.65 (23)	8.59±1.49 (37)	10.50±5.49 (4)

Growth. There was a treatment, generation, and interaction effect on larval growth (Table 19). F2 larvae exposed to the EtOH treatment were significantly smaller than F3 larvae exposed to EtOH, Low, and High treatments ($p<0.0001$; Fig 17). F2 larvae exposed to the Low treatment were smaller than F3 larvae exposed to Low, Medium, and High treatments ($p<0.0001$, $p<0.0001$, $p<0.016$; Fig 17). F2 larvae exposed to the Medium treatment were smaller than F3 larvae exposed to the Medium and High treatments ($p<0.0001$; Fig 17). F2 larvae exposed to the High treatment were smaller than F3 High treatment larvae ($p<0.0001$; Fig 17). For the third generation, Low treatment larvae were larger than Medium treatment larvae ($p<0.0001$), while Medium treatment larvae were smaller than High treatment larvae ($p=0.0001$; Fig 17, Table 18).

Table 19. Results of GLM examining the effects of treatment (EtOH Control, Low, Medium, High) and Generation (F2 and F3) on Growth of larval *P. promelas*. Significant effects are given in bold ($\alpha < 0.050$).

Effect	F	*df*	*P*
Treatment	13.2177	3	**<.0001***
Generation	175.5661	1	**<.0001***
Treatment *x* Generation	9.2915	3	**<.0001***
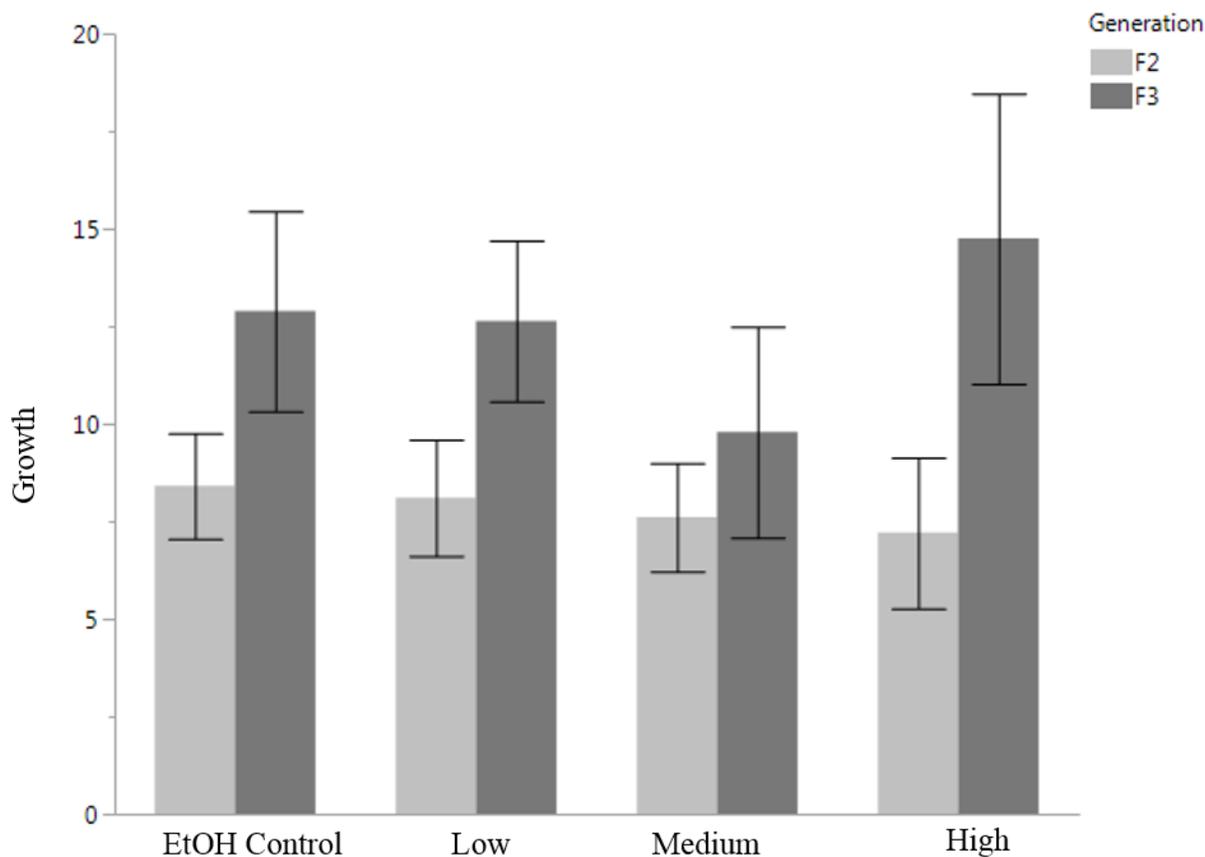
Figure 17. Bar Graph of Larval growth at 21 days post-hatch for F2 and F3 *P. promelas* with standard deviation error bars for larvae exposed to EtOH, Low, Medium, and High Treatments.

Feeding Assay. Based on the GLM, there was a significant difference of generation for the number of brine shrimp consumed by F2 and F3 larvae 21 days post-hatch (Table 20). F2 and F3 larvae exposed to EtOH ate more than F2 and F3 High treatment larvae (p=0.013, p=0.020; Fig 18). Low treatment F2 larvae ate less than F3 low treatment larvae (p=0.054; Table 14). F2 High treatment larvae at less than F2 and F3 EtOH and Medium treatment larvae (p=0.013, p=035, p=0.020, p=0.001; Fig 18). F2 High treatment larvae also ate less than F3 Low treatment larvae (p=0.002; Fig 18). F3 Low treatment larvae ate more than F2 Medium treatment larvae (p=0.045; Fig 18).

Table 20. Results of GLM examining the effects of treatment (EtOH Control, Low, Medium, High) and

Generation (F2 and F3) on feeding of larval *P. promelas*. Significant effects are given in bold ($\alpha < 0.050$).

| Effect	F	*df*	*P*
Treatment	1.557	3	0.669
Generation	8.783	1	**0.003***
Treatment *x* Generation	3.229	3	0.358

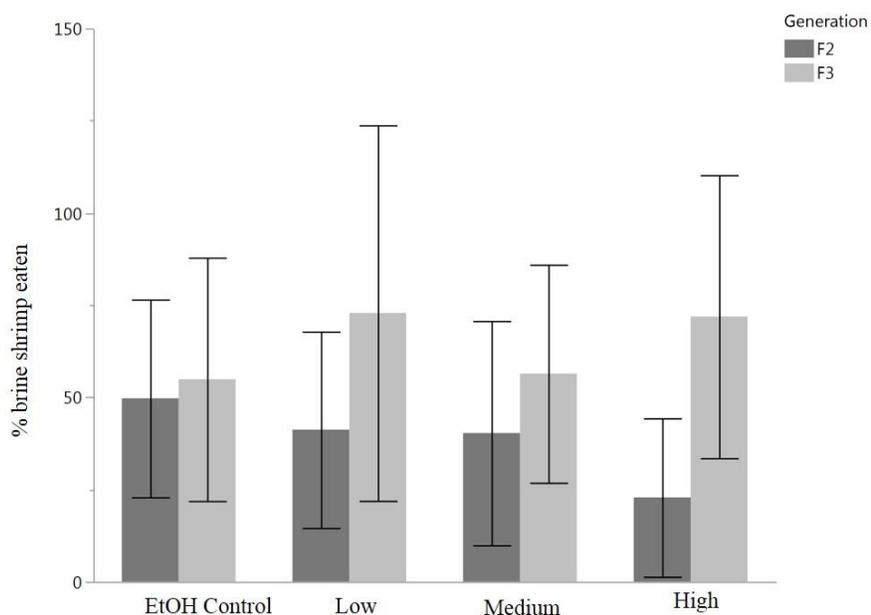*Figure 18*. Bar Graph of Larval Feeding at 21 days post-hatch for F2 and F3 *P. promelas* with standard

deviation error bars for larvae exposed to EtOH, Low, Medium, and High Treatments

2.4 Discussion

The present study is the first three-generational exposures of *P. pomelos* to an environmentally

derived urban CEC mixture and has provided additional evidence that life-cycle exposure of *P. promelas*

to low concentrations of CECs may alter fecundity and may have a greater impact on the success of the species than the effects of adult fish exposures would suggest. The study aimed to assess if (1) fish from subsequent generations would exhibit the same effects (adverse/therapeutic) when exposed and (2) whether subsequent generations would be more sensitive to exposure by eliciting a more severe biological response when exposed to a complex, urban CEC mixture.

Vitellogenin can be used as a predictor of fecundity in female fish, since it can determine the amount of energy attributed to developing embryos (Watanabee et al., 2016). Furthermore, the induction of vitellogenin in males exposed to CECs has led vitellogenin to be used as a biomarker for environmental estrogen exposure (Schwindt et al., 2007; Jobling et al., 1996). Fecundity, a measure of the number of eggs a female produces for a spawning event, can be influenced by the health condition (specifically length) of the female (Kooops et al,. 2004). In our experiment, we saw no differences in female vitellogenin concentrations However, we did see an increase in plasma vitellogenin concentrations of F1 males exposed to the High treatment compared to F1 EtOH and Low treatment males. To determine if the elevated vitellogenin concentrations have a significant impact on the fish population, fertility rates and sperm quality would need to be assessed in future studies. Exposure to single CECs generally produce decreases in fecundity, as Damman et al. (2011) and Weinberger and Klaper (2014) found in an exposure of *P. promeals* to estrone and fluoxetine, respectively. In mixture studies, the effects of exposures on reproduction have varied from no effect (Parrott & Bennie, 2009) to reduction in embryo production (Galus et al., 2013). Our study found low concentrations of the mixture increased fecundity for both F1 and F2 generations, corroborating our first hypothesis. This therapeutic hazardous effect was hypothesized to be due to the presences of pharmaceuticals in the mixture (i.e. the antidepressant – desvenlafaxine and the antibiotic - sulfamethoxazole). Brooks (2014) describe therapeutic hazards as the internal fish plasma level of a chemical equaling the therapeutic dose of a mammal ($THV = logP_{blood:water}$/human therapeutic dose). The therapeutic hazard helps explain therapeutic effects in

fish exposed to various CECs. Despite the overwhelming number of studies that have found adverse effects on fecundity when fish are exposed to various CECs, there have been few studies that have found therapeutic effects, which may be an artifact of the chemicals that have been studied in the past which seldom included pharmaceuticals. An increase in egg production was observed in *P. promelas* exposed to 0.32 ng/L of 17α-ethinylestradiol during a complete life-cycle exposure (Parrot & Blunt, 2005) and in medaka exposed to 10ug/L of ibuprofen (Han et al., 2010; Flipping et al., 2007). Similarly, Höger et al. (2006) found rainbow trout exposed to 15% wastewater treatment plant effluent for 32 days had an increase in fecundity. These studies support our findings, although additional testing on single and different mixtures should be conducted to determine which chemical or combination of chemicals were the cause of this therapeutic effect.

In addition to the therapeutic effect, we see a decrease in the cumulative mean number of eggs produced in females from the F1 to the F2 generation. Although we did not find significant differences in BCF, HSI, or GSI for individual treatments across the two generations, F2 females overall had a lower BCF and HSI values than F1 females. A decrease in BCF and HSI values generally indicates poor or stressful environments for fish (Mizanur et al., 2014). Our findings of a decrease in HSI of females over generations is consistent with the findings of Cripe et al. (2010) and our second hypothesis. In addition, the idea that fecundity is influenced by the health condition of the female (Kooops et al., 2004; Marshall et al., 1998) could be an additional explanation for the decrease in fecundity in the F2 generation. However, it should be noted, the EtOH also decreased in the cumulative mean number of eggs produced in F2. In the context of that information, it is possible that despite our minimal usage of EtOH, the F2 generation increased sensitivity to our carrier solvent. Other studies on zebrafish have found embryonic exposure to EtOH at 1-3% EtOH , as well as from 0-1% EtOH (vol/vol%) adversely altered adult behavior (Bailey et al., 2015; Fernandes & Gerlai, 2009).

Low treatment males in the F1 generation were phenotypically more dominant than F2 adult males from the EtOH, Medium, and High treatments based on secondary sex characteristic scores. Feminization of males has been a widely reported effect of CEC exposure in fish. Exposure of *P. promelas* to E1 (Damman et al., 2011) and EE2 (Parrott & Blunt, 2005) found males were demansculinized as E1 and EE2 concentrations increased. Smith (1978) found tubercle and dorsal pad development in male *P. promelas* co-occurred with the final stages of spermatogonia development. Based on Smith's (1978) findings, the change in secondary sex characteristics of males, in this present study, could then indicate F2 males are in worse reproductive condition than F1 males, corroborating both the first and second hypothesis of the present study.

Adverse effects on C-start performance of larval *P. promelas* exposed to CECs has been documented in previous studies (McGee et al., 2009; Painter et al., 2009). F3 larvae had a longer latency, slower escape velocity, and lower escape response than F2 larvae. Mauthner cells, located in the hindbrain, activate the C-start behavior (Eaton & Emberely, 1991). The exposure of fish to CECs can disrupt communication of the receptors and motor systems (Little & Brewer, 2001). These findings corroborate our second hypothesis that subsequent generations would be more sensitive to the CEC mixture. The adverse effect on C-start performance in subsequent generations, ultimately influences the ability of the fish to survive, leading to population level impacts.

F3 High treatment larvae were significantly larger than in any other treatment, which represents a different outcome when compared to the F2 High treatment larvae. These differences are thought to be density-dependent; where fish stocked at lower densities will grow faster and larger than fish stocked at higher densities (Lorenzen & Enberg, 2001). F3 Low treatment larvae ate more than F2 Low, Medium, and High treatments. This suggests a therapeutic effect on feeding when exposed to low CEC concentrations. A study by Brodin et al. (2013) found European perch exposed to environmentally relevant concentrations (1.8ug/L) of the benzodiazepine drug, lorazepam, were more active, asocial, and

foraged faster. Bisesi Jr, Bridges, and Klaine (2014) found hybrid bass exposed to the antidepressant venlafaxine (250ug/L and 500 ug/L) took longer to capture prey than unexposed bass. The differences in concentration of the pharmaceutical being used could explain the differences in responses seen. However, it must be noted, Brodin et al. (2013) also found fish exposed to 910ug/L of oxazepam also foraged faster than those exposed to 1.8ug/L. These differences in findings show that more testing should be done to determine if larvae exposed to a CEC mixture behave differently when foraging in the presence or absence of a predator.

Vacuolization of hepatocytes is thought to be an acute liver injury response (Nayak et al., 1996). F1 females exposed to Low treatments had greater vacuolization of hepatocytes than F2 Low treatment females. This finding is concurrent with the theory of acute liver injury response. The fish in the F1 generation are exposed as adults, while F2 generation fish are exposed for their entire lifecycle, leading to a potential resetting of the homeostasis point in the liver. CEC exposure can cause alterations in the distribution of gametogenic cell types (OECD, 2009). These changes can directly impact the survival of the population. GPA values for F1 and F2 generation fish found no treatment effects, showing no alterations in the distribution of gametogenic cell types. The lack of difference in GPA and GSI for F1 and F2 generation fish suggests that the fish were still reproductively fit. Exposure to CEC mixtures, particularly wastewater effluent, has also been found to have no effect on population dynamics in carp (Minarik et al., 2014).

The demasculization of F2 male *P. promelas,* increased delays in C-start responses of F3 larvae, and increased feeding rates of F3 larvae show a potential increased sensitivity to the CEC mixture in subsequent generations. On the other hand, the differences in fecundity, lack of significance in biological indices, and continued demasculization of F2 males represent similar effects across generations exposed to the same CEC mixture.

Complex mixtures are difficult to characterize, as it depends on the composition of the chemicals and species being targeted. Simple exposures of medaka to E2 and antiestrogens reduced some E2 effects (i.e. vitellogenin induction), but did not prevent adverse reproductive effects (Sun et al., 2009). Follow-up on how the individual chemical affect multiple generations would be useful in determining if these CECs work synergistically, antagonistically, or additively when in a mixture. In addition to the individual exposure, different combinations of a simple mixture would further help characterize the mixture effects.

The results of this study, shed light on the different effects complex CEC mixtures derived from water samples taken in the Laurentian Great Lakes Watersheds can adversely affect fish populations. In addition, it provides data to develop population models that can take into account different stressors, generational differences, energy reserves, and exposures to a diversity of CEC mixture.

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