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Life Cycle Exposure of Fathead Minnows to Complex Environmental Mixtures

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by

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Abstract

More than one-third of the Earth's freshwater is used for agricultural, industrial, and domestic purposes leading to the frequent co-occurrence of nitrate and mixtures of contaminants of emerging concerns in aquatic ecosystems. However, little is understood about the consequences of life-cycle exposure of fishes to these complex environmental mixtures. This project examined changes in physiology, performance, and reproduction in fathead minnows across three generations of exposure to agricultural and urban mixtures at environmentally relevant concentrations with an added stressor of nitrate. Exposure of adult fathead minnows in the first, but not second, generation to high nitrate concentrations resulted in a two-fold increase in egg production. In the second generation, the agricultural mixture enhanced fecundity in female fathead minnows above levels observed in EtOH control fish. Contrary to some published studies, neither nitrate nor estrogenic agricultural mixtures stimulated vitellogenin production in male fishes. In contrast, feminization (presence of the egg-yolk protein vitellogenin) was found in first generation males following exposure only to an urban chemical mixture independent of nitrate concentrations. Adult behavior does not appear to be affected regardless of treatment and generation. In contrast, larval behaviors, including predator avoidance performance and foraging efficiency, were both improved in higher nitrate treatments. Using an extended life-cycle fathead minnow exposure, we were able to improve our understanding of the consequences associated with long-term exposures to complex environmental mixtures. Overall, the observed effects of environmentally realistic mixtures were subtle and did neither follow a clear dose-response or matched effects observed in single compound exposures in the published literature. The complexity of interactions between multiple pollutant stressors observed in the current study highlight the need for additional such studies to ensure adequate assessment of environmental risk.

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Chapter 1: Literature Review and Problem Statement

Introduction

More than one-third of the Earth's freshwater is used for agricultural, industrial, and domestic purposes leading to the contamination of aquatic environments by a plethora of synthetic compounds including many contaminants of emerging concern (CECs). Given the only recent recognition of the potential environmental harm of CECs, little is known about the consequences of long-term exposure to CECs for aquatic life (Gomez et al., 2012; Loos et al., 2009).

When detected in the aquatic environment CECs are often found in complex mixtures (Elliott et al., 2017; Kolpin et al., 2002; Pal, Gin, Lin, & Reinhard, 2010,). The occurrence of specific chemicals in these mixtures is mostly driven by surrounding land use and subsequent inputs to the aquatic system through surface runoff and groundwater pollution (Fairbairn et al., 2018). Elliot and colleagues (2018), found that in the Great Lakes watershed CEC mixtures can be broadly characterized by land use into two major groupings: mixtures of agricultural and urban CECs, respectively.

Agricultural contaminants are becoming increasingly prominent in the environment as agricultural production has significantly increased from the 1960s (Rodvang & Simpkins 2001). As the need for increased food production continues to rise, the presence of herbicides and pesticides in the aquatic environment has become pronounced (Wauchope, 1978). These agricultural contaminants are often able to bind to soil particles and are transported through surface runoff into adjacent bodies of water during precipitation events (EPA, 2005). On the other hand, urban contaminants can include pharmaceuticals including hormones, personal care

products, and industrial chemicals. These chemicals frequently are not removed during the treatment process in wastewater treatment plants (WWTP) because they are not designed to remove these contaminants from effluent (Kolpin et al., 2002).

In addition to the presence of CECs in many agricultural and urban waterways, humans also have substantially altered the global nitrogen cycles over the past five decades ultimately increasing both the availability and the mobility of nitrogen over large regions of Earth (Camargo & Alonso 2006; Carpenter et al., 1998; Galloway & Cowling, 2002; Howarth et al., 2000; Vitousek et al., 1997). Consequently, in conjunction with natural sources, inorganic nitrogen can enter the aquatic environment through both point and nonpoint sources derived from human activities. The most prominent forms of dissolved inorganic nitrogen in aquatic environments are ammonium, nitrate, and nitrite (Camargo & Alonso 2006; Day et al., 1989; Howarth, 1988; Kinne, 1984; Rabalais & Nixon 2002; Wetzel 2001). As water rich with nitrate flows through landscapes it then enters riparian wetlands and head water streams, which can effectively and efficiently remove nitrogen. Therefore, making these key interfaces important in controlling nitrate export to downstream surface water (Burgin & Hamilton 2007; Peterson et al., 2001; Zedler, 2003). Typically, a rise in inorganic nitrogen concentration results in an increased number of primary producers which boosts organic production. However, ecosystems saturated in inorganic nitrogen that are unable to maintain the rate of assimilation have the potential to cause adverse effects on aquatic organisms (Camargo & Alonso 2006).

In the United States, nitrate levels have increased fivefold in the Midwest between 1945 and 1980 in intensively managed agricultural areas. Although this increase has slowed since 1980, the concentrations of nitrate have remained high throughout aquatic ecosystems in the Midwest (U.S. Rivers Show, 2015). While nitrate does occur naturally in ground water,

Dubvrovsky and colleagues (2010), found that nitrate concentrations over 1 mg/L indicate human activity. Knowing this, the EPA has set a maximum contaminant level for nitrate at 10 mg/L (EPA, 2020). High nitrate concentrations can lead to the formation of zones of low oxygen which can ultimately harm ecological habitats, recreational use, and fisheries. These high concentrations may also contribute to eutrophic conditions and stimulation of algal growth (Nitrogen Loading, n.d.). Nitrate recently became of interest to aquatic toxicologists due to its ability to alter endocrine function (Guillette & Edwards, 2005; Hamlin et al., 2008; Kellock et al., 2018).

CECs and nitrogenous contaminants are often studied in short-term single chemical exposures (Hoskins & Boone, 2017; Jensen et al., 2006; McGee et al., 2009) or field studies (Kidd et al., 2007; Palace et al., 2009; Schäfer et al., 2007), but little is understood about the long-term population relevant consequences of exposure to these complex mixtures.

Short-term single chemical exposures are a standard method for assessing biological consequences of exposure, but they are of limited ecological relevance as they inadequately depict the complexity of mixtures found in the environment and because of their short time frame (21 days). As it is impractical to test every individual contaminant present in an aquatic environment, an approach using complex, but environmentally realistic mixtures are needed to test the joint toxicity of these contaminants. Using the extended life-cycle approach of exposure with chemical mixtures proposed for this study addresses these factors. Furthermore, an extended life-cycle approach allows for comparison across exposure timeframes as the first generation is exposed only during their adult lifecycles while the second generation is exposed for the entirety of their lifecycle.

Field-based studies demonstrate ecosystem-wide alterations to contaminants, but they fail to identify the effects solely associated with the exposure to CECs. These field studies focus primarily on the biological effects of these CECs. Inevitably, there are variables that cannot be controlled, and questions left unanswered (Ankley & Villeneuve 2006). An extended life-cycle approach also allows for the control of many environmental variables while assessing endpoints, such as reproduction, which may serve as an indicator of whether a population-level threat may be present.

The current study addressed some of the knowledge gaps highlighted above by investigating the effects of complex chemical mixtures across multiple generations in a controlled fathead minnow (*Pimephales promelas*) laboratory study. The fathead minnow is a widely used model organism with frequent cycles of reproduction and a large number of offspring during a short life cycle. Furthermore, this species is hardy and able to withstand a wide range of basic water characteristics such as pH, dissolved oxygen, alkalinity, and temperature. Fathead minnows have been extensively studied and are well-understood fish models (Ankley & Villeneuve, 2006). However, the hardiness of this species may also hide subtle effects. This could sway the results into being less protective of more vulnerable species (Jorgenson et al., 2015).

I hypothesized that if exposed to environmentally relevant CEC mixtures over multiple generations, fathead minnows will demonstrate: 1) alterations to biological responses detrimental to both the organism (physiology and performance) and population level (reproduction), and 2) more severe biological responses will occur in generations two and three as they are exposed during their entire life-cycle as compared to generation one which was exposed only during their adult life. The objectives of the current study were to determine the

biological consequences of environmentally relevant CEC mixtures within the context of an extended life-cycle exposure utilizing fathead minnows as the model species. This approach will allow for greater environmental relevance due to the use of true environmental containment samples as well as the complete life-cycle exposure of our model organism.

Chapter 2: Complex Environmental Mixture Exposure Alters Biological Responses in First Exposed Generation

Introduction

As human populations have steadily increased over the past two hundred years, so has the presence of anthropogenic compounds in the aquatic environment. These compounds have the potential to affect the viability of aquatic ecosystems, highlighting the need for a better understanding of their effects on wildlife and human health alike. In addition to being ubiquitous in many anthropogenically influenced environments, these compounds are not found alone, but usually in complex mixtures ephemerally varying in composition and concentration. The majority of studies assessing the environmental threat of these compounds to-date have been brief in exposure duration and often focused on just one compound, or were conducted in field studies with limited opportunities to control for confounding variables. The goal of the current study was to determine how complex environmental mixtures (CEMs) may affect the health of fathead minnows and their populations using life-cycle exposures.

CEMs are groupings of compounds found in aquatic ecosystems that may have the potential to cause adverse effects at measured environmental concentrations. The mixtures of CEMs measured in an aquatic habitat commonly correlate to the surrounding land-use and the respective inputs to the aquatic ecosystem (Elliot et al., 2017; Elliot et al., 2018). Using an extensive existing data set of chemical occurrences in multiple Great Lakes tributaries, Elliot et al. (2017, 2018) identified recurring mixtures of CEMs correlated with the prevalence of agricultural practices and dense urban populations in the surrounding basin. Previous work using short term (days to weeks) and single compound exposures has determined that CEMs have the potential to cause behavioral changes (Garcia-Reyero et al., 2011; Kovacs et al., 2011; Martel et al., 2017; Martinovic et al., 2007; Schoenfuss et al., 2002), adverse physiological effects (Burki

et al., 2006; McMaster et al., 1991), and alterations to biological indices (McMaster et al., 1991; Tetreault et al., 2011).

In an analysis of over 300 water samples, Elliot and colleagues (2017, 2018) linked land use to the somewhat predictable occurrence of groupings of chemical compounds including pesticides, pharmaceuticals, personal care products, and industrial by-products. In addition to these predictable chemical mixtures, nitrogenous wastes are also ubiquitous in the Great Lakes and are known to be detrimental to aquatic life (Dove, 2009; Eimers & Watmough, 2016; Maguire et al., 2018). Over the last 5 decades, humans have substantially altered the global nitrogen cycle ultimately increasing both the mobility and availability of nitrogen (Camargo & Alonso, 2006; Carpenter et al., 1998; Galloway & Cowling, 2002; Howarth et al., 2000; Vitousek et al., 1997). Consequently, CEMs and nitrogen waste are entering aquatic ecosystems concurrently and may affect adversely impact the health of fish populations.

Among CEMs, several chemical classes are frequently reoccurring in chemical analyses and are known to impact aquatic life. Pesticides have been found to bind to soil particles that wash into bodies of water, ultimately, causing algal blooms and depleted dissolved oxygen concentrations (EPA, 2005). Pharmaceuticals have a wide variety of biochemical modes of action depending on the drug of interest, ultimately resulting in a wide variety of modifications from behavior to reproductive alterations (Daughton & Ternes, 1999). Balk and Ford (1999) found that personal care products, such as the fragrance galaxolide (HHCB), resulted in decreased growth of fish exposed for 21 days. Previous work on plasticizers, a group of wide-spread industrial by-product, found that not only are they carcinogenic, but may also be neurotoxic (Kim et al., 2011). Lastly, nitrogenous waste has recently gained renewed interest due

to its reported ability to disrupt endocrine function (Guillette & Edwards, 2005; Hamlin et al., 2008; Kellock, et al., 2018).

These contaminants are often studied in short-term single chemical exposures (Hoskins & Boone, 2017; Jensen et al., 2006; McGee et al., 2009) or field studies (Kidd et al., 2007; Palace et al., 2009; Schäfer et al., 2007), but little is understood about the long-term consequences of exposure to these chemicals or their effects in CEMs. Using an extended life-cycle CEM exposure in the current study addresses these knowledge gaps. Furthermore, a life-cycle exposure allows for comparison across exposure timeframes as the first generation is exposed only during their adult lifecycles while the second generation is exposed for the entirety of their lifecycle.

The overall goal of this study was to determine the effects of long-term exposure of CEMs on the health of fathead minnows and their populations. This approach provides greater environmental relevance due to its use of analytically derived environmental mixtures as well as the length of exposure encompassing all life stages of the exposed organism.

Materials and Methods

An extended life-cycle exposure was conducted using three generations of fathead minnows over a span of 285 days. The first exposed generation (F1) was obtained from a commercial laboratory fish culture (Environmental Consulting and Testing, Superior, WI) at sexual maturity (6 months old) and exposed for 60 days. Offspring were collected to propagate the F2 generation which was raised under continuous exposure to sexual maturity (6 months of age) and through an additional month of reproductive assessment. Offspring from F2 were then collected to propagate F3 which were raised for 21 days to make up three exposed generations (Figure 1). All procedures of animal care and use were approved by the St. Cloud State

University Institutional Animal Care and Use Committee (IACUC; approval permit #8-82) prior to the commencement of this study.

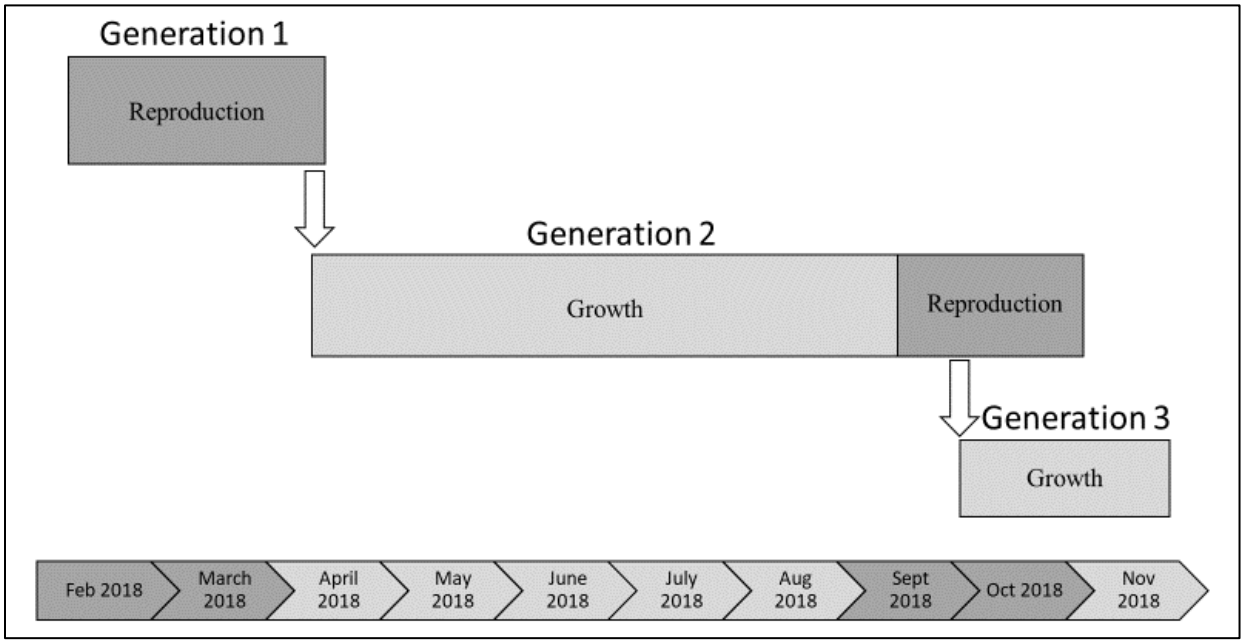

Figure 1. Extended life-cycle exposure timeline (duration: 285 days). Generation1 (F1) were obtained from a fish supplier and only exposed during adulthood. Generation 2 (F2) was exposed throughout their life cycle. Generation 3 (F3) was only maintained until the completion of juvenile behavioral trials (21 days post-hatch).

Chemistry. The chemical mixtures used in this laboratory study were derived from a matrix of occurrence and concentration data for several hundred organic contaminants in water samples collected from 24 of the Great Lakes' tributaries between 2010 and 2014 (Elliott et al., 2017). These grab samples were collected in the spring and summer from surface water spanning stretches of river associated with both agricultural and urban land use. In order to identify common co-occurring contaminants, water chemical analysis was conducted at the USGS National Water Quality Laboratory (Denver, CO) as previously described (Thomas et al., 2017). ArcGIS was utilized to characterize land use as urban or agricultural (Elliott et al., 2017). All contaminants detected in ≥30% of grab samples were included in a cluster analysis to determine

the composition of the laboratory mixture. Some families of compounds (for example, alkylphenols, estrogens) were combined into one chemical (4-nonylphenol, estrone, respectively) for the sake of logistical simplicity. The highest detected environmental concentration noted in the data matrix for each compound was used in the laboratory mixtures, with the resulting agricultural contaminants and urban contaminants mixtures and their concentrations illustrated in Figure 2 below.

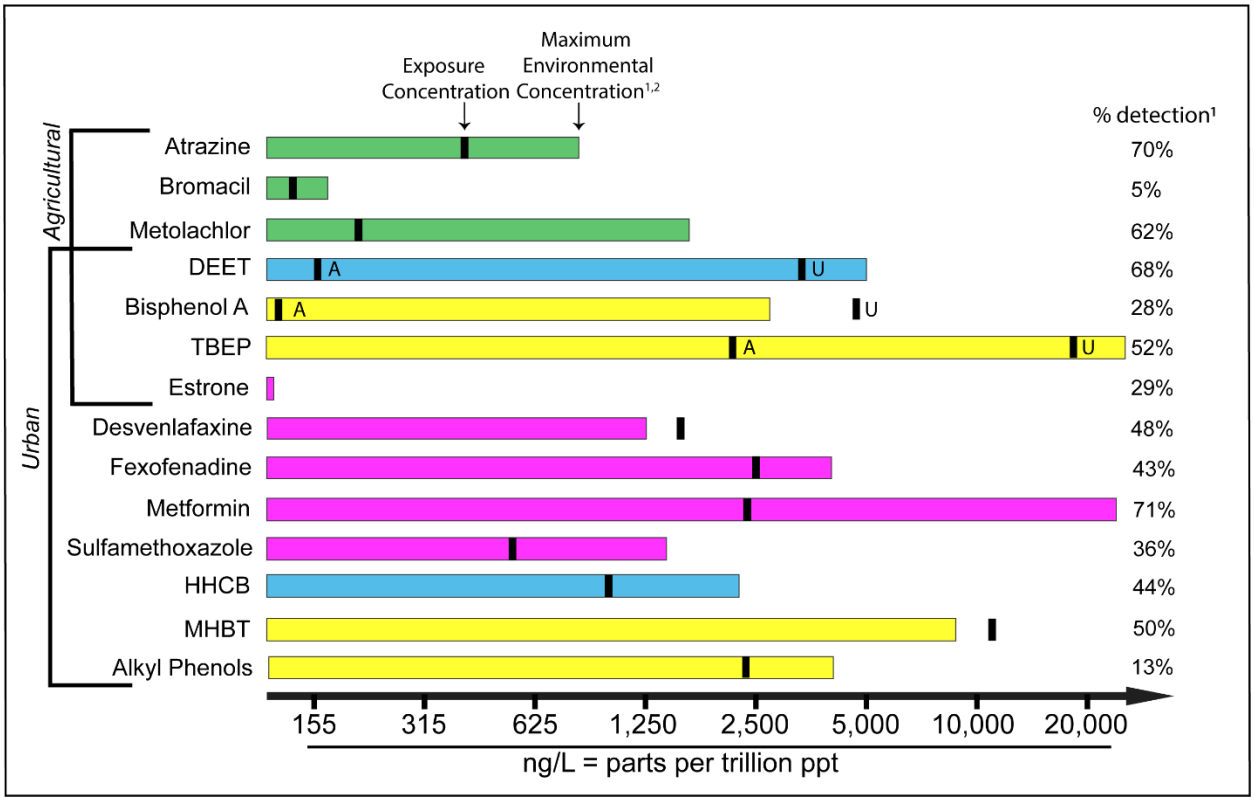

Figure 2. Chemical composition of mixtures broken down by chemical classes. Exposure concentrations [ng/L] indicated on the x-axis; maximum environmental concentrations documented in the scientific literature represented by colored bars. Chemical mixture composition is indicated by the brackets on the left. Chemical classes are depicted by colored bars where green represents pesticides, blue represents personal care products, yellow represents industrial by-products, and pink represents pharmaceuticals. Percent detection on the right side represents the percentage of total water samples these chemicals were detected in as reported in [1]Eliott et al, 2017, or [2]Bradley et al., 2017, respectively. DEET: N, N-Diethyl-meta-toluamide. TBEP: Tributoxyethyl Phosphate. HHCB: 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[g]-2-benzopyran. MHBT: 5-Methyl-1H-benzotriazole.

The concentrated stock solutions (in 100% EtOH) for the respective mixtures were assembled and validated by the USGS National Water Quality Laboratory. A solvent control (using 100% EtOH at the same concentration) and a blank well-water control were also employed. Two concentrations of nitrate were used in this study with the low concentration (5 mg/L) taken from one-half the maximum contaminant level which was set by the. EPA guidelines suggest a five-fold difference in exposure concentrations to ensure significance, placing the high concentration at 25 mg/L.

Water samples were collected in duplicate every nine days throughout the exposure in amber vials (20 mL Amber Borosilicate Vial, C&G Containers, Inc., Lafayette, LA) and frozen immediately (Appendix A). Water chemistry confirmation was conducted at USGS National Water Quality Laboratory to confirm exposure concentrations which are shown in supplemental table S1. Exposure aquaria were monitored every three days using an environmental multi-meter (Pro 1020, YSI Incorporated, Yellow Springs, OH) to measure dissolved oxygen (mg/L), temperature (°C), and pH. Water quality was monitored weekly using test strips (5 in 1 Water Quality Test Strips Cat. 27552-50, HACH, Loveland, CO) for total chlorine (ppm), free chlorine (ppm), general hardness (ppm), alkalinity (ppm), and pH. Nitrate concentrations were measured using a LabQuest (LabQuest 2, Vernier, Beaverton, OR) with a selective electrode probe (Nitrate Ion-Selective Electrode, Vernier, Beaverton, OR).

Experimental design. The Aquatic Toxicology Laboratory flow-through facility at St. Cloud State University was used to provide continuous exposure of fathead minnows over three successive generations spanning 9.5 months using an in-house dedicated well. The flow-through system constantly replenishes chemicals to the exposure system to minimize chemical loss due to chemical degradation or uptake. A daily three aquarium volume exchange rate was

targeted throughout the experiment. A pre-mixed solution of chemical stock prepared in 100% EtOH (4.5 mL) was dissolved in 10 L of DI water in an opaque carboy (3-gallon carboy glass, Northern Brewer, St. Paul, MN). Sodium Nitrate (Sigma Aldrich, Milwaukee, WI) (Low Nitrate = 20g and High Nitrate = 100g) was dissolved in 10 L of DI water in a second opaque carboy. The solutions were then pumped to stainless-steel mixing tanks above the exposure aquaria via a peristaltic pump (MasterFlex L/S 7519-06, Cole-Parmer, Vernon Hills, IL) at a rate of 2.5 mL/minute. Along with the chemical stock, temperature-adjusted well water (22-24°C) was pumped into the mixing tanks at a rate of 300 mL/minute. Flow rates were controlled using flow gauges (Valved Acrylic Flow Meter, Cole-Palmer, Vernon Hills, IL) to allow the chemical stock to mix prior to dispensing via gravity to the twelve exposure aquaria per treatment. Exposure water then flowed through opaque silicone tubing (Pentair Aquatic Ecosystems, Apopka, FL) controlled with the use of clamps (Screw Compressor Clamps, United Scientific Supplies, Inc., Waukegan, IL) into exposure aquaria (n = 12/treatment) (Tygon S3, Pentair Aquatic Ecosystems, Apopka, FL) under each head tank at a rate of 20 mL/minute, allowing for a turnover rate of approximately three total water exchanges per 24 hours. Each exposure aquaria housed one spawning pair of fathead minnows and held 10 L of water resulting in 12 spawning pairs per treatment (n = 12 males, n = 12 females per treatment). Blank well water control contained 16 exposure aquaria (n = 16).

Biological endpoints. Adult fathead minnows were anesthetized with neutral buffered 0.1% MS-222 (Argent Chemical Laboratories; Redmond, WA) prior to dissections (F1 – exposure day 60, F2 – exposure/life-cycle day 263). Fathead minnows were measured for length and wet mass (Ohaus Scout Pro 0.1g, Parsippany, NJ) to calculate condition factor (CF) as follows: CF = [body weight (g) / total length (mm)]. Male fathead minnows were analyzed by a

reviewer, blind to the fish's treatment to avoid bias, for a set of three secondary sexual characteristics (SSC). SSC consisted of a subjective 0 (absent/not visible) to 3 (present/ pronounced) rating each for tubercles, dorsal pad, and banding coloration as modified from Parrott et al. (2003). SSC was analyzed as the sum of the three characteristics on a scale from 0-9. The ovipositor length of female fathead minnows was measured (mm) using a digital caliper. Following these assessments, the tail of the minnows was then severed and a TRUEbalance blood glucose meter (Moore Medical LLC, Farmington, CT) was used to obtain a blood glucose concentration (mg/dL). Any reading below the detection limit of the reader (values below 20 mg/dL) was transformed as 50% of the lowest detection limit (i.e., 10mg/dL). Additional blood was then collected from the caudal vasculature using heparinized micro-hematocrit capillary tubes (Fisher Brand, Pittsburgh, PA) and centrifuged at 5,000 rpm for 5 min, after which time the percent hematocrit was recorded. Plasma was collected from the tubes and frozen for later vitellogenin (VTG) analysis. Laboratory analysis of plasma VTG was conducted using a competitive antibody-capture ELISA following Park et al. (1999) for plasma VTG quantification. Standard preparation and sample analysis followed previously described methods (Minarik et al., 2014). Laboratory analysis of plasma 11-ketotestosterone (11-KT) (Cayman Chemical 582751) and 17β-estradiol (E_2) (Cayman Chemical 501890) were conducted using the instructions provided with the respective kits in order to calculate 11-KT/E_2 ratio.

The liver and gonad of each fathead minnow were removed and weighed (Ohaus Scout 0.001g, Parsippany, NJ) for the calculation of the 1) hepatosomatic index (HSI) and 2) gonadosomatic index (GSI) using the following equations:

HSI = [liver weight (g) / total weight (g)] * 100

GSI = [gonad weight (g) / total weight (g)] * 100

Liver and gonad tissues were then placed in micromesh biopsy processing cassettes (Simport, Beloeil, QC, Canada) and preserved in 10% neutral buffered formalin for histological analysis.

Adult fathead minnows were analyzed during the reproduction periods (F1 – exposure day 56, 58, 61; F2 – life-cycle day 259, 261, 264) for three behaviors, including boldness, courtship, and nest defense. Boldness is measurement of a fishes willingness to approach a novel object in the tank, courtship is a measurement of the intensity of a male's courting behavior following the insertion of a female into the tank, and nest defense is a measurement of male aggression following the insertion of another male into the tank (the introduced male is confined in a clear glass tube to standardize this behavioral assessment). Further information regarding these measurements is available in previous work by Ward and colleagues (2017), and in Appendices B, C, and D. Adult behavioral trials were recorded using a GoPro 5 (GoPro, Inc., San Mateo, CA) and scored after the completion of the experiment according to Hasbay (2019).

During reproductive periods of both F1 (exposure days 21-60) and F2 (exposure days 233-263) generations, spawning tiles were checked daily to record fecundity (total number of eggs laid per female per treatment). Each clutch of larvae was monitored for growth, assessed for predator avoidance performance at 21 days (Appendix E), and underwent a feeding assay to test foraging efficiency on 21 days post-hatch. The predator avoidance performance, as previously described (McGee et al., 2009), determines a larval fathead minnow’s ability to respond to a perceived predatory stimulus in which latency (the duration for a larvae to respond after stimulus has been applied), escape velocity (the velocity at which an escaping larvae is swimming), escape angle (the angle at which a larva makes its escape path as compared to its original orientation), and total escape response (a calculation of a larvae’s ability to escape a perceived

stimulus based on escape velocity and escape angle) are assessed. 21-day old larval fathead minnows are also tested for their feeding efficiency (Appendix F). Larvae were placed in wells (1 larva per well) on a 6-well culture plate (Costar 3516, Corning Incorporated, Corning, NY) containing 10 mL of aerated well water. Larvae were administered a pre-determined amount (15 ± 1) of live hatched brine shrimp and allowed to consume as many as possible for a duration of 1 minute, after which the larvae were removed, and any remaining brine shrimp were counted and assessed as the percentage consumed.

After the removal of adult F1 fathead minnows (exposure day 60), F2 larvae were released from mesh baskets in each exposure aquarium to grow until sexual maturity and the start of the next reproduction period (exposure day 233). During this period, growth was assessed monthly by taking photographs of an aquarium and using ImageJ (1.50i) to determine the growth of juveniles within each aquarium by comparison to the metric grid underneath each aquarium. Growth measurements occurred during larval testing at age 21 days old, as well as at months 2, 3, and 4. During this growth period, larvae were culled on day 119 to maintain even density across aquariums and treatments while also avoiding delay in growth due to overcrowding of larvae.

Statistical analysis. Biological endpoints were analyzed using an ANOVA while growth and reproduction were analyzed using a repeated-measures ANOVA in JMP Pro 14 (SAS Institute). Significant differences between treatments were identified using Tukey's HSD test. The negative control (blank) was compared to the solvent control (EtOH alone) using a t-test. Unless noted otherwise in the results section, no significant differences existed between the negative and solvent control. Only the solvent (EtOH) controls were used in the subsequent statistical analysis of treatments as all treatments (other than the negative control) contained

ethanol as a carrier. VTG data were first normalized to the solvent control before statistical analysis was conducted. The statistical significance level was set at $p \leq 0.05$.

Results

Table 1. Behavioral performance of adult and larval fathead minnows across three subsequent generations. Mean and standard deviation for three adult (boldness; courtship; nest defense) and two larval (c-start [body length/ms]; foraging [% shrimp consumed in one minute]) behaviors assessed in adult male and larval fathead minnows, respectively. Sample size in the parenthesis. Detailed descriptions of each assay are found in the methods section of this manuscript and Ward et al. (2017). Bold numbers and superscripts signify significant differences between treatments.

		Treatment / Assay	F1-adult / F2-larvae			F2-adults / F3-larvae		
			Zero Nitrate	Low Nitrate	High Nitrate	Zero Nitrate	Low Nitrate	High Nitrate
Agricultural mixture	Adult	Boldness	0.0221±0.0034 (12)	0.132±0.33 (12)	0.0247±0.0063 (12)	0.0209±0.005 (11)	0.154±0.46 (12)	0.0264±0.0078 (12)
		Courtship	0.203±0.41 (8)	0.354±0.40 (8)	0.587±0.90 (12)	0.215±0.33 (11)	.315±0.35 (12)	0.0470±0.071 (12)
		Nest Defense	0.542±7.5 (8)	3.08±7.5 (8)	7.58±13.0 (12)	0.535±0.95 (11)	4.22±8.7 (12)	0.999±2.7 (11)
	Larvae	C-start [BL/ms]	**0.0080±0.006[a] (16)**	**0.0060±0.006[ac] (29)**	**0.0088±0.007[ac] (37)**	0.0038±0.004 (36)	0.0046±0.007 (36)	0.0063±0.007 (36)
		Foraging	54.8±0.29 (15)	76.1±0.31 (26)	72.5±0.28 (27)	**53.4±0.28[a] (40)**	52.6±0.27 (33)	**61.31±0.23[b] (35)**
EtOH control	Adult	Boldness	0.0215±0.0032 (12)	0.0210±0.0042 (11)	0.0212±0.0067 (11)	0.0241±0.0059 (12)	0.0212±0.004 (12)	0.0294±0.010 (12)
		Courtship	0.0845±0.12 (8)	0.240±0.30 (9)	0.633±0.48 (8)	0.150±0.3 (11)	0.081±0.2 (11)	0.270±0.52 (11)
		Nest Defense	1.48±0.95(8)	0.642±0.99(9)	0.489±0.57(9)	3.37±3.4(10)	0.345±0.67(11)	0.612±1.2(12)
	Larvae	C-start [BL/ms]	**0.0075±0.006[a] (30)**	**0.0064±0.005[ac] (29)**	**0.0089±0.006[ac] (39)**	0.0064±0.006 (39)	0.0064±0.006 (33)	0.0045±0.005 (35)
		Foraging	62.9±0.31 (30)	61.3±0.33 (38)	57.6±0.25 (38)	**47.1±0.30[a] (38)**	65.1±0.31 (32)	**71.2±0.23[b] (33)**
Urban mixture	Adult	Boldness	0.0242±0.0063 (12)	0.0191±0.0061 (9)	0.0191±0.0017 (11)	0.168±0.50 (12)	0.0260±0.009 (12)	0.0210±0.002 (12)
		Courtship	0.241±0.31 (10)	0.171±0.16 (7)	0.165±0.25 (12)	0.033±0.4 (10)	0.154±0.20 (12)	0.252±0.50 (10)
		Nest Defense	0.658±1.1 (10)	0.909±1.5 (7)	5.201±14.0 (12)	1.02±3.0 (9)	0.905±1.0 (12)	0.795±1.9 (10)
	Larvae	C-start [BL/ms]	**0.0060±0.005[b] (37)**	**0.0034±0.003[bc] (36)**	**0.0053±0.005[bc] (37)**	0.0040±0.004 (42)	0.0045±0.005 (36)	0.0053±0.005 (36)
		Foraging	69.9±0.32 (32)	53.1±0.28 (36)	61.1±0.29 (36)	**63.8±0.27[a] (42)**	59.5±0.31 (38)	**61.2±0.29[b] (38)**

Males. F1 and F2 adult males showed no significant difference in boldness, courtship, and nest defense performance as confirmed by an ANOVA. Total escape response, the cumulative measure of escape performance in larval fish, was significantly different between CEMs and between nitrate concentrations in F2 generation fathead minnow larvae. Exposure to agricultural CEMs resulted in an increased escape response as compared to urban treatments ($p = 0.0012$). Similarly, EtOH treatments showed a significant greater escape response as compared to urban treatments ($p = 0.0007$). Furthermore, nitrate exposure at both concentrations resulted in an increased escape response when compared to the treatments containing no nitrate ($p = 0.0360$). Interestingly, this effect disappeared in F3 larvae. Foraging efficiency was unaffected by any F2 exposure, while F3 larvae displayed an increase in foraging efficiency in high nitrate concentration as compared to the treatments with no addition of nitrate ($p = 0.0237$). More specifically, foraging efficiency for the EtOH treatment with high nitrate was significantly increased as compared to the zero nitrate EtOH treatment ($p = 0.0099$).

Table 2. Biological endpoints of adult male fathead minnows across two subsequent generations. Mean and standard deviation for nine adult endpoints assessed in adult male fathead minnows, respectively. Most endpoints are unitless. Sample size in the parenthesis. Detailed descriptions of each assay are found in the methods section of this manuscript. Bold numbers and superscripts signify significant differences.

	Treatment / Assay	*F1 Males*			*F2 Males*		
		Zero Nitrate	Low Nitrate	High Nitrate	Zero Nitrate	Low Nitrate	High Nitrate
Agricultural mixture	Survival	57%	57%	86%	92%	91%	100%
	TL/SL Ratio	1.20±0.060 (8)	1.22±0.030 (8)	1.21±0.020 (12)	**1.22±0.090^{a} (11)**	**1.21±0.030^{b} (11)**	**1.21±0.030^{a} (16)**
	CF	1.35±0.27 (8)	1.24±0.19 (8)	1.23±0.16 (12)	1.56±0.17 (11)	1.62±0.29 (11)	1.35±0.22 (16)
	GSI	1.59±0.82 (8)	1.61±1.3 (8)	1.34±0.73 (12)	1.68±0.81 (11)	1.84±0.42 (11)	1.41±0.70 (16)
	HSI	1.58±0.67 (8)	1.48±0.45 (8)	1.93±0.59 (12)	2.59±1.1 (11)	2.01±1.3 (11)	2.45±1.34 (16)
	Glucose [mg/dL]	38.50±19.0 (8)	38.50±14.7 (8)	48.18±16.3 (11)	**62.45±10.2^{a} (11)**	**61.00±15.9^{b} (11)**	58.87±21.0 (16)
	Hematocrit [%]	40.3±8.1 (8)	**36.8±10.0^{c} (8)**	**37.7±8.4^{c} (12)**	54.1±9.4 (11)	55.1±9.4 (11)	49.4±10.0 (16)
	11-KT/E-2 Ratio	543.62±457.1 (4)	570.50±606.7 (5)	294.94±409.5 (9)	380.67±369.3 (9)	288.27±339.8 (8)	462.45±358.4 (13)
	SSC	4.29±1.1 (7)	3.63±1.6 (8)	3.67±1.9 (12)	**5.09±1.8^{a} (11)**	4.55±1.8 (11)	**2.69±2.0^{b} (16)**
EtOH control	Survival	79%	57%	43%	92%	100%	100%
	TL/SL Ratio	1.22±0.020 (11)	1.21±0.040 (8)	1.24±0.030 (6)	**1.21±0.030^{a} (12)**	**1.05±0.090^{b} (13)**	**1.14±0.11^{a} (19)**
	CF	1.23±0.21 (11)	1.77±1.2 (8)	2.04±1.2 (6)	1.53±0.21 (12)	1.55±0.24 (13)	1.45±0.22 (19)
	GSI	1.12±0.061 (11)	1.42±0.058 (8)	1.97±1.2 (6)	2.18±2.9 (12)	1.68±0.069 (13)	2.25±3.7 (19)
	HSI	1.59±0.72 (11)	1.55±0.54 (8)	1.65±0.45 (6)	2.39±1.1 (12)	2.58±1.17 (13)	2.13±1.5 (19)
	Glucose [mg/dL]	46.89±12.5 (9)	40.57±9.74 (7)	47.50±7.69 (6)	**78.08±28.4^{a} (12)**	**56.08±12.6^{b} (13)**	53.16±16.7 (19)
	Hematocrit [%]	**58.7±20.0^{b} (3)**	**33.6±6.9^{bc} (8)**	**40.3±7.9^{bc} (6)**	47.5±6.8 (11)	50.4±13.0 (13)	49.4±8.7 (18)
	11-KT/E-2 Ratio	492.91±0.00 (1)	243.04±430.19 (5)	940.37±895.6 (5)	581.62±867.2 (11)	852.92±972.9 (11)	454.49±508.2 (13)
	SSC	3.50±1.9 (10)	3.38±1.8 (8)	3.83±2.0 (6)	**4.82±2.4^{a} (11)**	3.46±1.5 (13)	**3.11±2.3^{b} (18)**
Urban mixture	Survival	71%	57%	79%	100%	100%	77%
	TL/SL Ratio	1.21±0.030 (10)	1.21±0.020 (8)	1.21±0.030 (11)	**1.21±0.030^{a} (16)**	**1.14±0.10^{b} (16)**	**1.21±0.02^{a} (14)**
	CF	1.25±0.10 (10)	1.25±0.12 (8)	1.21±0.23 (11)	1.37±0.23 (16)	1.44±0.25 (16)	1.52±0.28 (14)
	GSI	1.47±0.050 (10)	1.46±0.64 (8)	1.41±0.57 (11)	1.36±0.93 (16)	2.21±3.21 (16)	1.55±0.53 (14)
	HSI	1.94±0.71 (10)	1.59±0.41 (8)	2.20±0.93 (11)	2.11±0.98 (16)	1.47±0.70 (16)	2.70±0.65 (14)
	Glucose [mg/dL]	43.20±9.21 (10)	43.25±6.25 (8)	39.78±12.0 (9)	**58.80±16.7^{a} (15)**	**49.75±13.2^{b} (16)**	59.15±17.9 (13)
	Hematocrit [%]	**36.2±10.0^{ac} (10)**	**34.6±4.9^{ac} (8)**	**35.7±7.9^{ac} (11)**	47.4±10.2 (16)	47.5±10.0 (16)	48.8±7.9 (14)
	11-KT/E-2 Ratio	95.06±110.0 (8)	286.28±324.0 (7)	642.92±636.9 (9)	414.30±492.7 (15)	361.56±358.0 (14)	277.09±438.0 (13)
	SSC	3.40±1.8 (10)	4.14±1.4 (7)	4.00±2.0 (11)	**3.87±2.7^{a} (15)**	3.47±2.2 (15)	**4.17±2.4^{b} (12)**

In first generation males, there was no significant difference in survival, TL/SL ratio, SSC, CF, GSI, HIS, 11-KT/E-2 ratio, or glucose. However, hematocrit was significantly lower in the urban treatment ($p = 0.0099$) when compared to the EtOH control. In addition, treatments containing zero nitrate had significantly higher hematocrit values as compared to treatments with low nitrate ($p = 0.0024$) and high nitrate ($p = 0.0358$). More specifically, hematocrit was significantly decreased in the agricultural treatment with high nitrate ($p = 0.0140$), agricultural treatment with low nitrate ($p=0.0148$), and EtOH with low nitrate ($p = 0.0029$) when compared to the zero nitrate EtOH treatment. Similarly, hematocrit was significantly decreased in the urban without the addition of nitrate ($p = 0.0081$), urban with low nitrate ($p = 0.0050$), and urban with high nitrate ($p = 0.0055$) when compared to the zero nitrate EtOH treatment. In contrast, F2 males were found to have no significant differences in survival, CF, GSI, HIS, 11-KT/E-2 ratio, and hematocrit. However, F2 males exposed to treatments with low nitrate concentrations displayed decreased TL/SL ratios when compared to treatments containing high nitrate ($p = 0.0028$) and treatments with no addition of nitrate ($p < 0.001$). Despite that, minnows exposed to treatments containing no addition of nitrate had significantly increased prevalence of SSC versus those exposed to treatments containing high nitrate concentrations (0.0236). Additionally, glucose concentrations were significantly increased in males not exposed to nitrate when compared to males exposed to low nitrate concentrations ($p = 0.0311$).

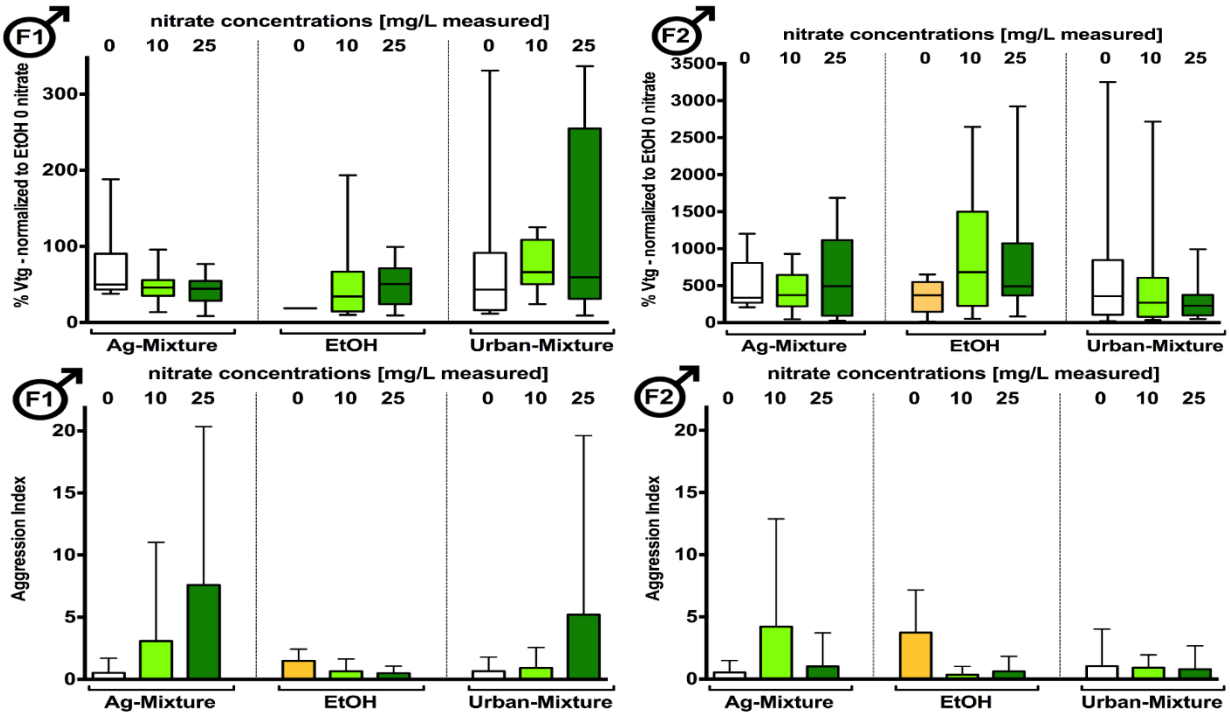

Figure 3. Adult male biomarkers. F1 (left) and F2 (right) VTG (top) and aggression (bottom). VTG depicted as % VTG normalized to EtOH. Aggression measured according to Hasbay (2019). Treatment displayed on lower x-axis with nitrate concentration displayed on upper x-axis.

F1 males were found to have a significant increase in percent VTG in the urban treatment when compared to the agricultural treatment regardless of nitrate concentration ($p = 0.0543$). The aggression index did not exhibit significant differences between any treatments. Interestingly, F2 males were found to have no significant differences in VTG concentrations or aggression index regardless of nitrate concentration ($p = 0.0543$).

Table 3. Behavioral performance of adult and larval fathead minnows across three subsequent generations. Mean and standard deviation for three adult (boldness; courtship; nest defense) and two larval (c-start [body length/ms]; foraging [% consumed]) behaviors assessed in adult male and larval fathead minnows, respectively. Sample size in the parenthesis. Detailed descriptions of each assay are found in the methods section of this manuscript. Bold numbers and superscripts signify significant differences.

			Zero Nitrate	Low Nitrate	High Nitrate	Zero Nitrate	Low Nitrate	High Nitrate
Agricultural mixture	Adult	Boldness	0.0221±0.0034 (12)	0.132±0.33 (12)	0.0247±0.0063 (12)	0.0209±0.005 (11)	0.154±0.46 (12)	0.0264±0.0078 (12)
		Courtship	0.203±0.41 (8)	0.354±0.40 (8)	0.587±0.90 (12)	0.215±0.33 (11)	.315±0.35 (12)	0.0470±0.071 (12)
		Nest Defense	0.542±7.5 (8)	3.08±7.5 (8)	7.58±13.0 (12)	0.535±0.95 (11)	4.22±8.7 (12)	0.999±2.7 (11)
	Larvae	C-start	**0.0080±0.006^{a} (16)**	**0.0060±0.006^{ac} (29)**	**0.0088±0.007^{ac} (37)**	0.0038±0.004 (36)	0.0046±0.007 (36)	0.0063±0.007 (36)
		Foraging	54.8±0.29 (15)	76.1±0.31 (26)	72.5±0.28 (27)	**53.4±0.28^{a} (40)**	52.6±0.27 (33)	**61.31±0.23^{b} (35)**
EtOH control	Adult	Boldness	0.0215±0.0032 (12)	0.0210±0.0042 (11)	0.0212±0.0067 (11)	0.0241±0.0059 (12)	0.0212±0.004 (12)	0.0294±0.010 (12)
		Courtship	0.0845±0.12 (8)	0.240±0.30 (9)	0.633±0.48 (8)	0.150±0.3 (11)	0.081±0.2 (11)	0.270±0.52 (11)
		Nest Defense	1.48±0.95(8)	0.642±0.99(9)	0.489±0.57(9)	3.37±3.4(10)	0.345±0.67(11)	0.612±1.2(12)
	Larvae	C-start	**0.0075±0.006^{a} (30)**	**0.0064±0.005^{ac} (29)**	**0.0089±0.006^{ac} (39)**	0.0064±0.006 (39)	0.0064±0.006 (33)	0.0045±0.005 (35)
		Foraging	62.9±0.31 (30)	61.3±0.33 (38)	57.6±0.25 (38)	**47.1±0.30^{a} (38)**	65.1±0.31 (32)	**71.2±0.23^{b} (33)**
Urban mixture	Adult	Boldness	0.0242±0.0063 (12)	0.0191±0.0061 (9)	0.0191±0.0017 (11)	0.168±0.50 (12)	0.0260±0.009 (12)	0.0210±0.002 (12)
		Courtship	0.241±0.31 (10)	0.171±0.16 (7)	0.165±0.25 (12)	0.033±0.4 (10)	0.154±0.20 (12)	0.252±0.50 (10)
		Nest Defense	0.658±1.1 (10)	0.909±1.5 (7)	5.201±14.0 (12)	1.02±3.0 (9)	0.905±1.0 (12)	0.795±1.9 (10)
	Larvae	Foraging	69.9±0.32 (32)	53.1±0.28 (36)	61.1±0.29 (36)	**63.8±0.27^{a} (42)**	59.5±0.31 (38)	**61.2±0.29^{b} (38)**

Female. The only behavior performed by and analyzed for in female adult fathead minnows was boldness for which no significant difference was found in F1 or F2 fish. F2 larval total escape response showed significant differences between treatments and nitrate concentrations. Agricultural treatments had an increased escape response as compared to the urban treatments ($p = 0.0012$). Similarly, EtOH treatments showed a significantly greater

escape performance as compared to the urban treatments ($p = 0.0007$). Furthermore, exposure to high nitrate concentrations resulted in an increased escape response when compared to treatments with low nitrate concentrations ($p = 0.0030$). In addition, escape performance was significantly increased in low nitrate treatments when compared with treatments containing no nitrate ($p = 0.0360$). Interestingly, F3 larvae showed no significant difference in total escape response. When assessing foraging efficiency, F2 larvae showed no significant differences in feeding efficiency. In contrast, F3 larvae displayed an increase in foraging efficiency in high nitrate treatments as compared to the treatments with no addition of nitrate ($p = 0.0237$). More specifically, foraging efficiency for the EtOH treatment with a high concentration of nitrate was significantly greater as compared to the zero nitrate EtOH treatment ($p = 0.0099$).

Table 4. Biological endpoints of adult female fathead minnows across two subsequent generations. Mean and standard deviation for nine adult endpoints assessed in adult female fathead minnows, respectively. Sample size in the parenthesis. Detailed descriptions of each assay are found in the methods section of this manuscript. Bold numbers and superscripts signify significant differences.

	Treatment / Assay	*F1 Females*			*F2 Females*		
		Zero Nitrate	Low Nitrate	High Nitrate	Zero Nitrate	Low Nitrate	High Nitrate
Agricultural mixture	Survival	63%	88%	75%	**75%^{a}**	**93%^{b}**	**100%^{b}**
	TL/SL Ratio	1.22±0.030 (10)	1.21±0.030 (14)	1.20±0.030 (12)	1.22±0.080 (9)	1.23±0.020 (16)	1.23±0.020 (13)
	CF	1.32±0.14 (10)	1.45±0.42 (14)	1.34±0.22 (12)	1.36±0.14 (9)	1.33±0.14 (16)	1.29±0.15 (13)
	GSI	12.24±5.98 (10)	11.26±5.69 (14)	10.50±3.66 (12)	9.47±2.5 (9)	11.43±5.18 (16)	11.72±5.28 (13)
	HSI	2.94±0.71 (10)	2.33±1.12 (14)	4.56±4.2 (12)	2.52±1.6 (9)	2.18±1.3 (16)	2.14±1.2 (13)
	Glucose [mg/dL]	**68.89±38.2^{a} (9)**	**63.25±20.5^{a} (12)**	**77.25±18.1^{a} (12)**	71.11±23.3 (9)	64.31±16.5 (16)	66.08±34.2 (12)
	Hematocrit [%]	39.4±12.0 (10)	42.3±6.8 (14)	43.3±11.0 (12)	45.6±3.2 (5)	52.0±8.5 (15)	52.5±9.5 (12)
	11-KT/E-2 Ratio	0.34±0.3 (3)	0.24±0.0 (1)	N/A (0)	3.95±0.0 (1)	0.33±0.2 (4)	23.94±44.7 (5)
	OVI	**3.99±0.99^{a} (10)**	**3.64±0.70^{b} (14)**	**3.47±0.93^{a} (12)**	**4.05±0.72^{a} (9)**	**4.19±0.57^{a} (12)**	**3.37±0.77^{a} (11)**
EtOH control	Survival	63%	69%	56%	**92%^{a}**	**100%^{b}**	**100%^{b}**
	TL/SL Ratio	1.21±0.040 (10)	1.21±0.030 (11)	1.20±0.020 (9)	1.24±0.050 (15)	1.23±0.040 (13)	1.23±0.040 (10)
	CF	1.20±0.26 (10)	1.37±0.16 (11)	1.10±0.15 (9)	1.35±0.16 (15)	1.42±0.18 (13)	1.43±0.19 (10)
	GSI	10.56±4.91 (10)	11.62±3.70 (11)	9.71±4.4 (9)	10.96±5.00 (15)	14.65±6.87 (13)	15.12±4.9 (10)
	HSI	2.01±1.4 (10)	2.88±2.0 (11)	1.75±0.74 (9)	1.87±1.1 (15)	2.78±1.12 (13)	2.24±1.0 (10)
	Glucose [mg/dL]	**41.70±25.7^{b} (10)**	**55.18±14.4^{b} (11)**	**39.25±12.9^{b} (8)**	**86.07±28.5^{a} (15)**	66.77±15.5 (13)	60.40±18.0 (10)
	Hematocrit [%]	33.7±11.0 (10)	39.6±9.6 (11)	37.6±12.0 (8)	60.5±14.0 (13)	51.3±13.0 (13)	50.5±15.0 (10)
	11-KT/E-2 Ratio	N/A (0)	26.16±0.00 (1)	N/A (0)	84.62±187.0 (5)	0.51±0.0 (1)	0.27±0.2 (2)
	OVI	**3.47±0.93^{a} (10)**	**3.70±0.83^{b} (11)**	**3.41±0.50^{a} (9)**	**3.56±0.58^{b} (11)**	**3.30±0.58^{b} (13)**	**3.29±0.79^{b} (8)**
Urban mixture	Survival	69%	81%	56%	**82%^{a}**	**100%^{b}**	**100%^{b}**
	TL/SL Ratio	1.23±0.090 (11)	1.19±0.040 (13)	1.21±0.030 (9)	1.24±0.020 (11)	1.25±0.020 (14)	1.22±0.070 (13)
	CF	1.37±0.34 (11)	1.31±0.29 (13)	1.22±0.32 (9)	1.30±0.11 (11)	1.29±0.13 (14)	1.34±0.20 (13)
	GSI	10.58±5.49 (11)	11.73±6.33 (13)	10.84±4.99 (9)	13.58±4.95 (11)	12.36±3.34 (14)	10.74±7.57 (13)
	HSI	2.32±1.0 (11)	1.87±0.83 (13)	2.76±2.4 (9)	4.40±6.4 (11)	2.27±1.1 (14)	3.47±6.1 (13)
	Glucose [mg/dL]	66.81±12.9 (11)	52.77±19.5 (13)	49.63±29.6 (8)	**47.33±9.15^{b} (9)**	58.31±22.2 (13)	73.58±29.7 (12)
	Hematocrit [%]	40.5±7.2 (11)	37.1±8.5 (13)	46.0±12.0 (9)	50.9±19.0 (8)	53.3±6.8 (12)	54.2±10.0 (10)
	11-KT/E-2 Ratio	15.56±21.9 (2)	0.47±0.0 (1)	N/A (0)	5.06±1.9 (2)	41.65±70.3 (3)	8.38±10.0 (3)
	OVI	**3.52±0.50^{a} (11)**	**3.66±0.50^{b} (13)**	**3.05±0.59^{a} (9)**	3.42±0.54 (9)	3.53±0.44 (12)	3.64±0.63 (12)

First generation females were found to have no significant difference in survival, total length/standard length, condition factor, GSI, HSI, hematocrit, or 11-KT/E-2 ratio. Blood glucose, an indicator of metabolic stress, displayed a significant increase in the first-generation agricultural treatment when compared to the EtOH control ($p = 0.0002$). In addition, first generation females were found to have increased ovipositor length in treatments with low nitrate when compared to treatments with high nitrate ($p = 0.0359$) and treatments with zero nitrate ($p = 0.0523$). On the other hand, second generation females displayed no significant differences in total length/standard length, condition factor, GSI, HSI, hematocrit, or 11-KT/E-2 ratio. Second generation females were found to display an increase in survival in treatments containing high nitrate ($p = 0.0206$) and low nitrate ($p = 0.0381$) when compared to treatments with no addition of nitrate. Furthermore, blood glucose was higher in the EtOH control with no addition of nitrate when compared to the urban treatment with no addition of nitrate ($p = 0.0048$). Lastly, ovipositor length was significantly increased in the agricultural treatment when compared to the EtOH control ($p = 0.0086$). More specifically, the agricultural treatment with low nitrate had an increased ovipositor length when compared to the EtOH treatment with low nitrate ($p = 0.0181$).

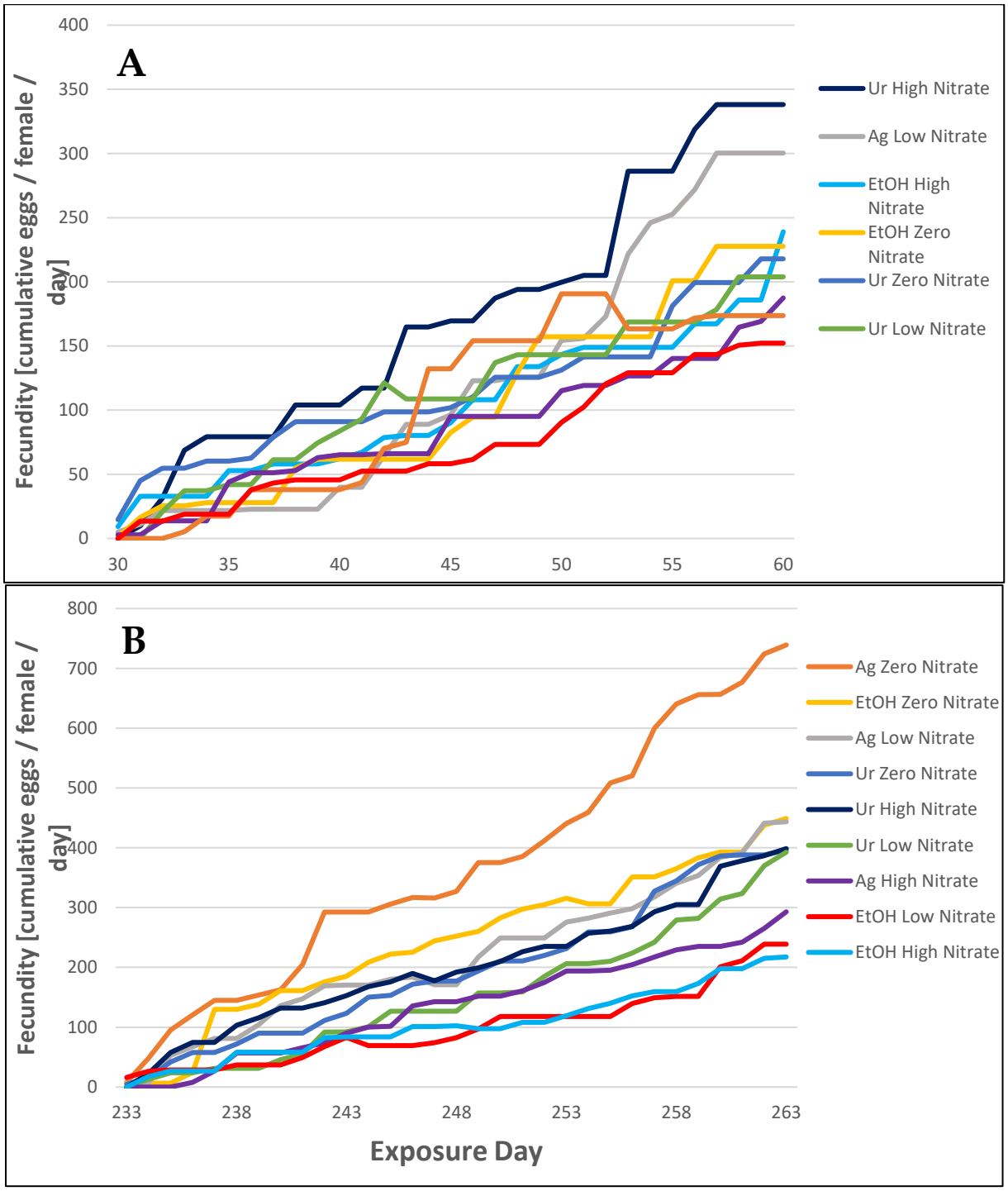

Figure 4. F1 (A) and F2 (B) fecundity depicted as the cumulative mean number of eggs per female per day. Data were adjusted by day for each treatment to account for any mortality within treatments.

Analysis was completed through a repeated-measures ANOVA using the square root of egg number. Significant differences between treatments were identified using Tukey's HSD test. F1 females had no significant differences in egg production. Interestingly, F2 females displayed an increase in egg production in the agricultural treatment when compared to the EtOH treatments, regardless of nitrate concentration ($p = 0.0092$). Furthermore, treatments containing high nitrate ($p = 0.0083$) and low nitrate ($p = 0.0300$) concentrations had a significant decrease in egg production when compared to treatments containing no addition of nitrate.

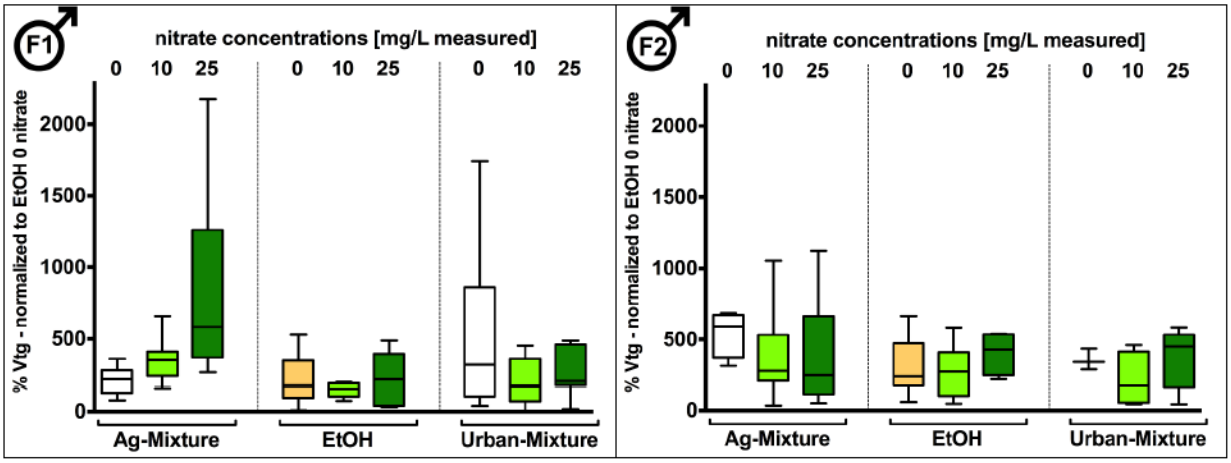

Figure 5. F1 (left) and F2 (right) VTG depicted as % VTG normalized to EtOH. Treatments are shown on the lower x-axis with nitrate concentration shown on the upper x-axis.

F1 females exposed to agricultural treatment displayed a significant increase in percent VTG as compared to all EtOH treatments regardless of nitrate concentration ($p = 0.0285$). More specifically, the agricultural treatment with high nitrate was significantly increased as compared to agricultural treatment with no addition of nitrate ($p = 0.0132$), EtOH treatment with low nitrate ($p = 0.0203$) and zero nitrate EtOH ($p = 0.0346$), and urban treatment with low nitrate ($p = 0.0092$). On the other hand, F2 females displayed no significant differences in percent VTG between treatments or nitrate concentrations.

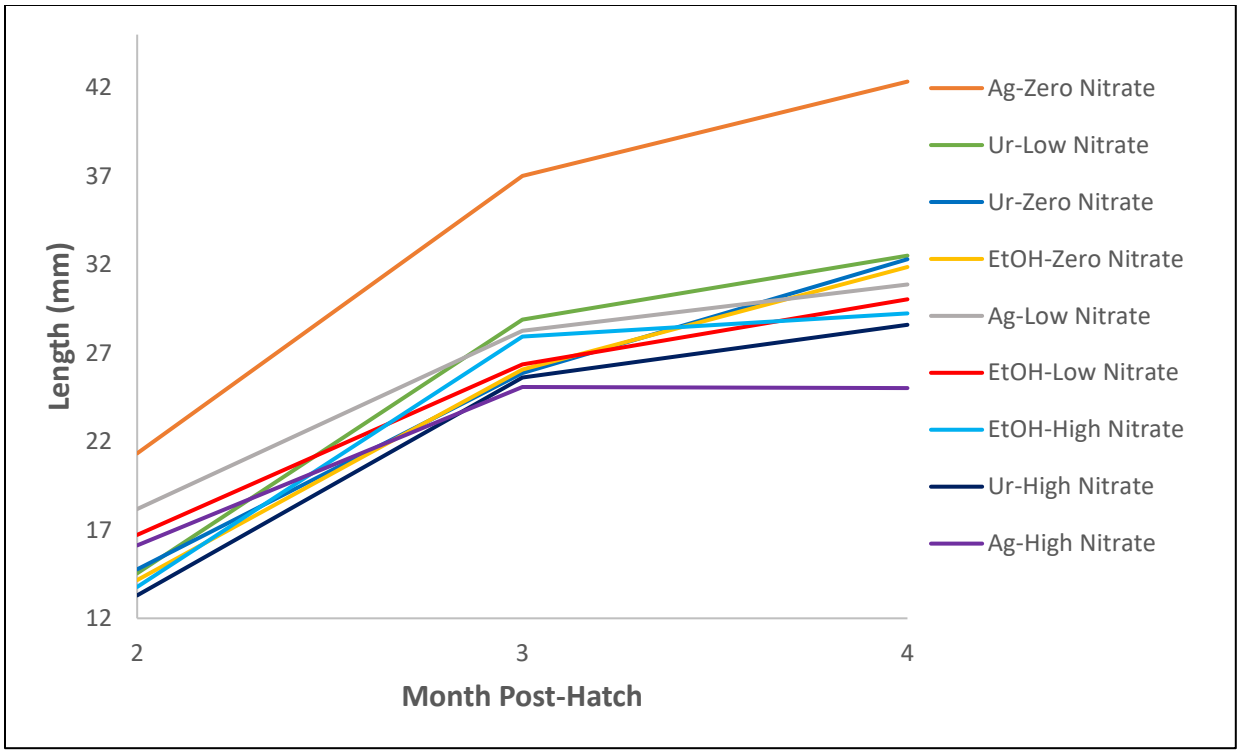

Figure 6. Second generation larval growth measured in mm.

Larvae. Second generation larvae were found to have significantly increased growth in the agricultural treatment at every month of development when compared to the zero nitrate EtOH control ($p < 0.0001$) and urban treatment ($p < 0.0001$). All other treatments followed the same growth trend and displayed no significant differences. Treatments containing low nitrate ($p = 0.0190$) and high nitrate ($p < 0.0001$) had significantly decreased growth when compared to treatments containing no addition of nitrate. Furthermore, treatments containing high nitrate had significantly decreased growth when compared to treatments containing low nitrate concentrations ($p < 0.0001$).

Discussion

The objective of this study was to assess the effects of CEMs across life-cycle exposures on the health of fathead minnows and their populations. Utilizing a life-cycle exposure allowed for a more detailed assessment of the environmental consequences associated with long-term exposure to CEMs. In general, F1 exposed fathead minnows demonstrated more severe biological responses, highlighting the potential for adaptation across generations.

Previous work by Weber and Spieler (1994) identified that chemical contaminants might act upon neural networks resulting in changes to normal behaviors by altering physiological responses. Alterations to normal behaviors following exposure to agricultural and urban contaminants have been found to change swimming performance and survival (Scott & Sloman, 2004). The boldness (Pellegrini et al., 2010), courtship (Cole & Smith, 1987), and nest defense (Sargent, 1989; Unger, 1983) behaviors have been well documented in previous work. Although significant increases in the presence of SSC in F2 exposed males, which is a beneficial trait for both courtship and nest defense behaviors (Danylchuk & Tonn, 2001), no changes in performance occurred in the presence of any treatment group or nitrate concentration. These results indicated no perceived harm in the exposed male's ability to successfully mate and maintain a nesting site.

Glucose has been previously identified as an indicator of stress (Bevelhimer et al., 2014; Carvalho & Fernandes, 2008; Thomas et al., 2017) due to upregulation in response to organism exposure to contaminants. An upregulation in glucose commonly correlates to increased stress. Glucose concentrations remained consistently highest in the EtOH control treatment, indicating no increasing stress response across all treatments and nitrate concentrations. Furthermore, hematocrit, the percent of blood made of red blood cells, is commonly used as an indicator of an

organism's ability to maintain osmotic homeostasis and respiratory function (Barham et al., 2006; Davies, 1987). Similarly, to glucose, hematocrit percentage remained highest in treatments with no addition of nitrate indicating the organism is unaffected in their respiratory function as a result of CEM or nitrate exposure. While Kramer and colleagues (1998) demonstrated that male fish exposed to estrogenic compounds have higher sensitivity in their hematocrit response than female conspecifics (1998), the composition of CEMs, and the presence of nitrate appears to cause no respiratory stress to male or female fathead minnows in any treatment.

Female fathead minnows develop an extension of the gonadal papilla (ovipositor) utilized during mating to direct the deposit of eggs (Danylchuk & Tonn, 2001). Ovipositor length is frequently used as an early indicator of feminization in both male and female fathead minnows. In females, exposure to estrogenic contaminants may lead to early development or increased length of ovipositors. Similarly, in male fathead minnows, estrogenic exposure may lead to the development of an ovipositor (Parrott & Wood, 2002). An increase in the length of the ovipositor in females is believed to correlate with a decrease in fecundity according to Price (1972). An increase in ovipositor length can be seen in both first- and second-generation females without an impact on fecundity suggesting that there was no detrimental impact on the ability of female fathead minnows to reproduce successfully. These results were further confirmed when assessing female fecundity as there were no significant decreases in egg production in either generation. Although, F2 females in the agricultural treatment with no addition of nitrate were found to have a significant increase in egg production, which likely corresponds to the increase in growth displayed in that treatment.

The production of VTG in male fish has frequently been used for an indicator of estrogenic exposure with the possibility of leading to feminization of male fathead minnows

(Harries et al., 1996, Harries et al., 1997; Purdom et al., 1994). An upregulation of VTG may not pose an immediate adverse effect on the health of the male fish; however, it may indicate the improper allocation of energy resources as this plasma protein is energetically costly to biosynthesis and as the protein is of no functional value in male oviparous animals. This effect occurred in F1 males who displayed an increase in percent VTG in the urban treatment when compared to the EtOH control, which was not unexpected as the urban treatment is the most estrogenic treatment. F1 females displayed an increase in percent VTG in the agricultural treatment, which corresponded to an increase in egg production. Despite the effects observed in F1 generation male and female fathead minnows, no significant differences in VTG concentrations were found in F2 generation males or females, suggesting a possible adaptive response to chronic exogenous estrogen exposure.

Adult endpoints were frequently less sensitive than those associated with the developing fathead minnow larvae. The slightest of alterations to a larva's predator-avoidance behavior has the potential to be catastrophic at the population level, as previously described by Kidd and colleagues (2007) and Rearick et al. (2018). Interestingly, an increase in nitrate concentration corresponded with an enhanced total escape response in the current study.

The observed significant differences in juvenile growth in the F2 generation larvae resulted in fish in the agricultural treatment with no addition of nitrate being larger than larvae in all other treatments. This effect may be a result of the lower density in these larval rearing aquaria (due to the low reproductive rate and survival of fish in this treatment) and not a direct treatment effect.

Previous work has found that exposure to individual environmental contaminants have the potential to result in behavioral changes (Garcia-Reyero et al., 2011; Kovacs et al., 2011;

Martel et al., 2017; Martinovic et al., 2007; Schoenfuss et al., 2002), adverse physiological effects (Burki et al., 2006; McMaster et al., 1991), and alterations to biological indices (McMaster et al., 1991; Tetreault et al., 2011). Despite these reports and many others in the scientific literature, there is a substantial gap in the knowledge of the effects of CEM exposure, highlighting the importance of conducting long-term complex mixture exposures as was performed in the current study. Only conducting these time and labor intense long-term studies will lead to an improved understanding of the environmental consequences of environmentally realistic mixtures.

Conclusion

An analysis of a total life-cycle assessment of CEMs highlights the potential of complex environmental mixtures of anthropogenic chemicals to propagate detrimental biological consequences, not only at the organismal level but also at the population level, as evident by changes in egg production by female fathead minnows in the current study. Studies of this magnitude are crucial for the direct interpretation of chemical mixture effects and an understanding of generational effects. Life-cycle studies also highlight differences in responses to exposures between generations. Several mechanisms may explain these observed differences. The time frame of exposure for F1 generation fish only encompassed adulthood, while the F2 generation adults had been exposed continuously during embryogenesis and sexual differentiation. The prolonged duration of exposure may have resulted in adaptation across generations, whereas the exposure conditions may have selected for F2 generation fish with lower sensitivity to chemicals in the CEMs. These effects, if confirmed, cannot be observed in single chemical and short-term exposures.

The relative ease of single chemical and short-term exposure is understood, and the importance of these studies should not be disregarded. Although, in the aquatic environment, the likelihood of an organism being exposed to an individual contaminant or for just a short period of time is unlikely. Rather, these organisms are exposed to complex chemical mixtures where interactions between contaminants may propagate unforeseeable effects. The interactions between chemicals have the potential to be synergistic, additive, antagonistic, or reversed, according to Jackson and colleagues (2016), ultimately, lessening or amplifying the single effect of a contaminant. Indeed, these authors (Jackson et al., 2016) concluded that only in a minority of mixture studies were the combined effects greater than for the individual compounds in the mixtures.

Overall, the current study identifies and addresses knowledge gaps that previously inhibited a comprehensive assessment of the environmental consequences of life-cycle exposures to environmentally realistic, complex mixtures of anthropogenic contaminants in aquatic environments. The approach taken in the current study, while logistically difficult, provides great environmental relevance, and others should be encouraged to follow with further investigations into the chronic effects of environmentally derived complex chemical mixtures.

Chapter 3: Overall Conclusions

Contamination of aquatic ecosystems with anthropogenic contaminants has been an environmental and human health concern for many years, especially following the industrial revolution. In fact, in 1969, the Cuyahoga River in Ohio burst into flames due to chemical waste released into the waterway, highlighting the fact that industrial pollution was severely impacting waterways. Interestingly enough, this may have been the last time that the Cuyahoga caught on fire, but it was not the first. This river burned at least thirteen times before public authorities acknowledged the pollutant problem (Stradling & Stradling, 2008). Highly visible and unequivocal events such as the burning of the Cuyahoga River ultimately inspired the creation of the Clean Water Act in 1972 designed towards reducing pollution. Despite this, in 2009, the U.S. Environmental Protection Agency reported that more than half of United States stream ecosystems are facing severe pollution damage (EPA, 2009).

Over the past decade, as a result of climate change and nutrient fed “dead zones” in freshwater and marine ecosystems, increasing attention has been paid to understanding the effects of chemical mixtures and pollution in aquatic environments. Commonly contaminants are studied at high concentrations in hopes of finding toxicity levels, which can be beneficial for helping to place water quality limitations. Furthermore, contaminants are frequently studied in short-term exposures due to the relative ease that corresponds with a study of this length. Although, the concentrations used are often not realistically representing what is found in aquatic environments and a short-term study does not encapsulate the real consequences of exposure through an entire lifecycle. Utilizing accurate, environmental concentrations of these contaminants over multiple generations is ultimately going to most accurately represent the biological consequences associated with exposure to CEMs in the aquatic environment.

The need for shifts in agricultural and urban practices necessary to prevent continued degradation of aquatic health is highlighted by the effects of CEMs on aquatic species. Current management practices in both agricultural and urban settings cater to the growing world population. These same practices are adversely impacting aquatic organisms, in turn, posing a threat to human health. Therefore, it is necessary that more considerable attention is being paid to best management practices, pollutant reduction and green chemistry which would precipitate a shift to more biodegradable chemicals. Potential changes in management practices could include altering the application of herbicides in agricultural settings, initiating proper disposal programs for unused pharmaceuticals, or ensuring the outflows for storm and sanitary sewers are separated. Initiating changes in management practices would help reduce pollution of agricultural, urban, and nitrate contaminants at the source.

Unfortunately, reducing pollution at the source by initiating changes in management practices is only a small piece of the puzzle that is environmental health. Anthropogenic climate change causing warming water temperatures and seasonal changes acts as an additional stressor to aquatic organisms. This added stressor may make aquatic organisms more susceptible to adverse effects corresponding to prolonged exposure to CEMs or may facilitate the movement of pollutants into aquatic habitats as a result of more severe and frequent precipitation events. The additive effects of stress due to climate change and stress due to CEM exposure may result in increased mortality, altering population dynamics.

In order to identify the possibility of altering population dynamics, as highlighted in Chapter 2, there is a need for life-cycle exposure to CEMs. The current utilization of short-term exposures of single contaminants or even of mixtures may not fully suffice to understand the effects of exposure in a real aquatic ecosystem. Ultimately bringing to light the need to adapt

exposure protocols to capture all windows of development, including embryo, larval, juvenile, and adult life stages, present in a life-cycle exposure that would otherwise be missed in short-term exposure. Results from this study, and studies of this magnitude, may benefit those responsible for making decisions regarding alterations to management practices to preserve biodiversity in aquatic ecosystems.

References

Ankley, G. T., & Villeneuve, D. L. (2006). The fathead minnow in aquatic toxicology: Past, present and future. *Aquatic Toxicology*, *78*, 91-102. https://doi.org/10.1016/j.aquatox.2006.01.018

Balk, F., & Ford, R. A. (1999). Environmental risk assessment for the polycyclic musks, AHTN and HHCB. II. Effect assessment and risk characterisation. *Toxicology Letters*, *111*(1-2), 81-94. https://doi.org/10.1016/S0378-4274(99)00170-8

Barham. W. T., Smit, G. L., & Schoonbee, H. J. (2006). The heamatological assessment of bacterial infection in Rainbow Trout, Salmo gairdneri Richardson. *Journal of Fish Biology*, *17*(3), 275-281. https://doi.org/10.1111/j.1095-8649.1980.tb02761.x

Bevelhimer, M. S., Adams, S. M., Fortner, A. M., Greeley, M. S., & Brandt, C. C. (2014). Using ordination and clustering techniques to assess multimetric fish health response following a coal ash spill. *Environmental Toxicology and Chemistry, 33*(8), 1903-1913. https://doi.org/10.1002/etc.2622

Bradley, P. M., Journey, C. A., Romanok, K. M., Barber, L. B., Buxton, H. T., Foreman, W. T., … & Villeneuve, D. L. (2017). Expanded target-chemical analysis reveals extensive mixed-organic-contaminant exposure in U.S. streams. *Environmental Science and Technology*, *51*(9), 4792-4802. https://doi.org/10.1021/acs.est.7b00012

Burgin, A. J., & Hamilton, S. K. (2007). A review of nitrate removal pathways have we overemphasized the role of denitrification in aquatic ecosystems? *Frontiers in Ecology and the Environment, 5*(2), 89-96.

Burki, R., Vermeirssen, E. I. M., Korner, O., Joris, C., Burkhardt-Holm, P., & Segner, H. (2006). Assessment of estrogenic exposure in brown trout (Salmo trutta) in a Swiss midland

river: integrated analysis of passive samplers, wild and caged fish, and vitellogenin mRNA and protein. *Environmental Toxicology and Chemistry*, *25*(8), 2077-2086.

Camargo, J. A., & Alonso, Á. (2006). Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. *Environment International, 32,* 831-849. https://doi.org/10.1016/j.envint.2006.05.002

Carpenter, S. R., Caraco, N. F., Correll, D. L., Howarth, R. W., Shaarpley, A. N., & Smith, V. H. (1998). Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecological Application, 8,* 559-568.

Carvalho, C. dos S., & Fernandes, M. N. (2008). Effect of copper on liver key enzymes of anaerobic glucose metabolism from freshwater tropical fish Prochilodus lineatus. *Comparative Biochemistry and Physiology-A Molecular and Integrative Physiology*, *151*(3), 437-442. https://doi.org/10.1016/j.cbpa.2007.04.016

Cole, K. S., & Smith, R. J. F. (1987). Male courting behaviour in the fathead minnow, Pimephales promelas. *Environmental Biology of Fishes*, *18*(3), 235-239.

Danylchuk, A. J., & Tonn, W. M. (2001). Effects of social structure on reproductive activity in male fathead minnows (Pimephales promelas). *Behavioral Ecology*, *12*(4), 482-489. https://doi.org/10.1093/beheco/12.4.482

Daughton, C. G., & Ternes, T. A. (1999). Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environmental Health Perspectives*, *107*(SUPPL. 6), 907-938. https://doi.org/10.1289/ehp.99107s6907

Davies, P. E. (1987). Physiological, anatomic and behavioural changes in the respiratory system of Salmo gairdneri rich. on acute and chronic exposure to chlorothalonil. *Comparative*

Biochemistry and Physiology Part C: Comparative Pharmacology, *88*(1), 113-119. https://doi.org/10.1016/0742-8413(87)90054-5

Day, J. W., Hall, C. A. S., Kemp, W. M., & Yanez-Arancibia, A. (1989). *Estuarine ecology*. New York: John Wiley and Sons.

Dove, A. (2009). Long-term trends in major ions and nutrients in Lake Ontario. *Aquatic Ecosystem Health & Management*, *12*(3), 281-295. https://doi.org/https://doi.org/10.1080/14634980903136388

Dubvrovsky, N., Burow, K., Clark, G., Gronberg, J., Hamilton, P., Hitt, K., … & Wilber, W. (2010). Nutrients in the nation's streams and groundwater, 1992-2004. *USGS Circular*, *1350*, 174.

Eimers, M. C., & Watmough, S. A. (2016). Increasing nitrate concentrations in streams draining into Lake Ontario. *Journal of Great Lakes Research*, *42*(2), 356-363.

Elliott, S. M., Brigham, M. E., Lee, K. E., Banda, J. A., Choy, S. J., Gefell, D. J., … & Jorgenson, Z. G. (2017). Contaminants of emerging concern in tributaries to the Laurentian Great Lakes: I. Patterns of occurrence. *PLoS ONE*, *12*(9), 1-21. https://doi.org/10.1371/journal.pone.0182868

Elliott, S. M., Brigham, M. E., Kiesling, R. L., Schoenfuss, H. L., & Jorgenson, Z. G. (2018). Environmentally relevant chemical mixtures of concern in waters of U.S. tributaries to the Great Lakes environmental management environmentally relevant chemical mixtures of concern in waters of United States tributaries to the Great Lakes. *Integrated Environmental Assessment and Management*, pp. 1-10. https://doi.org/10.1002/ieam.4041

EPA. (2005). Protecting water quality from agricultural runoff. Retrieved from https://www.epa.gov/sites/production/files/2015-09/documents/ag_runoff_fact_sheet.pdf

EPA. (2009). National rivers and streams assessment 2008-2009 results. Retrieved from https://www.epa.gov/national-aquatic-resource-surveys/national-rivers-and-streams-assessment-2008-2009-results

EPA. (2020). National primary drinking water regulations. Retrieved from https://www.epa.gov/ground-water-and-drinking-water/national-primary-drinking-water-regulations

Fairbairn, D. J., Elliott, S. M., Kiesling, R. L., Schoenfuss, H. L., Ferrey, M. L., & Westerhoff, B. M. (2018, August). Contaminants of emerging concern in urban stormwater: Spatiotemporal patterns and removal by iron-enhanced sand filters (IESFs). *Water Research, 145*, 332–345. https://doi.org/10.1016/j.watres.2018.08.020

Galloway, J. N., & Cowling, E. B. (2002). Reactive nitrogen and the world: 200 years of change. *AMBIO, 31*(2), 64-71. https://doi.org/10.1579/0044-7447-31.2.64

Garcia-Reyero, N., Lavelle, C. M., Escalon, B. L., Matrinovic, D., Kroll, K. J., Sorenson, P. W., & Denslow, N. D. (2011). Behavioral and genomic impacts of a wastewater effluent on the fathead minnow. *Aquatic Toxicology, 101*(1), 38-48. https://doi.org/https://doi.org/10.1016/j.aquatox.2010.08.014

Gomez, M. J., Herrera, S., Sole, D., Garcia-Calvo, E., & Fernandez-Albe, A. R. (2012). Spatio-temporal evaluation of organic contaminants and their transformation products along a river basin affected by urban, agricultural and industrial pollution. *Science of the Total Environment, 420*, 134-145. https://doi.org/10.1016/j.scitotenv.2012.01.029

Guillette, A., Jr, L. J., & Edwards, T. M. (2005). Is nitrate an ecologically relevant endocrine disruptor in vertebrates? *Integrative and Comparative Biology, 45*, 19-27.

Hamlin, H. J., Moore, B. C., Edwards, T. M., Larkin, I. L. V, Boggs, A., High, W. J., … & Guillette, Jr., L. J. (2008). Nitrate-induced elevations in circulating sex steroid

concentrations in female Siberian sturgeon (*Acipenser baeri*) in commercial aquaculture. *Aquaculture*, *281*, 118-125. https://doi.org/10.1016/j.aquaculture.2008.05.030

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Routledge, E. J., … & Tylor, T. (1996). A survey of estrogenic activity in United Kingdom inland waters. *Environmental Toxicology and Chemistry*, *15*(11), 1993-2002. https://doi.org/10.1897/1551-5028(1996)015<1993:ASOEAI>2.3.CO;2

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J. P., …& Zaman, N. (1997). Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environmental Toxicology and Chemistry*, *16*(3), 534-542. https://doi.org/10.1897/1551-5028(1997)016<0534: EAIFUK>2.3.CO;2

Hasbay, U. (2019). "From single chemicals to complex mixtures": Effect of contaminants of emerging concern on three life stages of Pimephales Promelas. *Culminating Projects in Biology*, *41*.

Hoskins, T. D., & Boone, M. D. (2017). Atrazine feminizes sex ratio in Blanchard's cricket frogs (*Acris blanchardi*) at concentrations as low as 0.1μg/L. *Environmental Toxicology and Chemistry*, *37*(2), 427-435. https://doi.org/10.1002/etc.3962

Howarth, R. W. (1988). Nutrient limitation of net primary production in marine ecosystems. *Annual Review of Ecology and Systematics, 19,* 89-110.

Howarth, R. W., Anderson, D., Cloern, J., Elfring, C., Hopkinson, C., Lapointe, B., … & Walker, D. (2000). Nutrient pollution of coastal rivers, bays, and seas. *Issues in Ecology, 7,* 1-5.

Jackson, M. C., Loewen, C. J. G., Vinebrooke, R. D., & Chimimba, C. T. (2016). Net effects of multiple stressors in freshwater ecosystems: A meta-analysis. *Global Change Biology*, *22*(1), 180-189. https://doi.org/10.1111/gcb.13028

Jensen, K. M., Makynen, E. A, Kahl, M. D., & Ankley, G. T. (2006). Effects of the feedlot contaminant 17alpha-trenbolone on reproductive endocrinology of the fathead minnow. *Environmental Science & Technology*, *40*(9), 3112-3117. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16719119

Jorgenson, Z. G., Buhl, K., Bartell, S. E., & Schoenfuss, H. L. (2015). Do laboratory species protect endangered species? Inter-species variation in responses to 17b-estradiol, a model endocrine active compound. *Archives of Environmental Contamination and Toxicology 68*, 204-215.

Kellock, K. A., Moore, A. P., & Bringolf, R. B. (2018). Chronic nitrate exposure alters reproductive physiology in fathead minnows. *Environmental Pollution*, *232*, 322-328.

Kidd, K. A., Blanchfield, P. J., Mills, K. H., Palace, V. P., Evans, R. E., Lazorchak, J. M., & Flick, R. W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(21), 8897-8901. https://doi.org/10.1073/pnas.0609568104

Kim, J. W., Isobe, T., Chang, K. H., Amano, A., Maneja, R. H., Zamora, P. B., … & Tanabe, S. (2011). Levels and distribution of organophosphorus flame retardants and plasticizers in fishes from Manila Bay, the Philippines. *Environmental Pollution*, *159*(12), 3653-3659. https://doi.org/10.1016/j.envpol.2011.07.020

Kinne, O. (1984). *Marine ecology*. London: John Wiley and Sons.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., & Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A National Reconnaissance. *Environmental Science & Technology*, *36*(6), 1202-1211.

Kovacs, T. G., Martel, P. H., O'Connor, B. I., Parrott, J. L., McMaster, M. E., Van Der Kraak, G. J., … & Hewitt, M. L. (2011). Kraft mill effluent survey: Progress toward best management practices for reducing effects on fish reproduction. *Environmental Toxicology and Chemistry2*, *30*(6), 1421-1429. https://doi.org/https://doi.org/10.1002/etc.526

Kramer, V. ., Miles-Richardson, S., Pierens, S. ., & Giesy, J. (1998). Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (Pimephales promelas) exposed to waterborne 17β-estradiol. *Aquatic Toxicology*, *40*(4), 335-360. https://doi.org/10.1016/S0166-445X(97)00060-X

Loos, R., Gawlik, B. M., Locoro, G., Rimaviciute, E., Contini, S., & Bidoglio, G. (2009). EU-wide survey of polar organic persistent pollutants in European river waters. *Environmental Pollution*, *157*, 561-568. https://doi.org/10.1016/j.envpol.2008.09.020

Maguire, T. J., Wellen, C., Stammler, K. L., & Mundle, S. O. C. (2018). Increased nutrient concentrations in Lake Erie tributaries influenced by greenhouse agriculture. *Science of The Total Environment*, *633*, 433-440. https://doi.org/https://doi.org/10.1016/j.scitotenv.2018.03.188

Martel, P. H., O'Connor, B. I., Kovacs, T. G., van der Heuvel, M. R., Parrott, J. L., McMaster, M. E., … & Hewitt, M. L. (2017). The relationship between organic loading and effects

on fish reproduction for pulp mill effluents across Canada. *Environmental Toxicology and Chemistry*, *51*(6), 3499-3507. https://doi.org/https://doi.org/10.1021/acs.est.6b05572

Martinovic, D., Hogarth, W. T., Jones, R. E., & Sorenson, P. W. (2007). Environmental toxicology. *Environmental Toxicology and Chemistry*, *26*(2), 271-278.

McGee, M. R., Julius, M. L., Vajda, A. M., Norris, D. O., Barber, L. B., & Schoenfuss, H. L. (2009). Predator avoidance performance of larval fathead minnows (Pimephales promelas) following short-term exposure to estrogen mixtures. *Aquatic Toxicology*, *91*(4), 355-361. https://doi.org/https://doi.org/10.1016/j.aquatox.2008.12.002

McMaster, M. E., Van der Kraak, G. J., Portt, C. B., Munkittrick, K. R., Sibley, P. K., Smith, I. R., & Dixon, D. G. (1991). Changes in hepatic mixed-function oxygenase (MFO) activity, plasma steroid levels and age at maturity of a white sucker (*Catostomus commersoni*) population exposed to bleached kraft pulp mill effluent. *Aquatic Toxicology*, *21*(3-4), 199-217. https://doi.org/https://doi.org/10.1016/0166-445X(91)90073-I

Minarik, T., Vick, J., Schultz, M. M., Bartell, S., Martinovic-Weigelt, D., Rearick, D., & Schoenfuss, H. (2014). On-site exposure to treated wastewater effluent has subtle effects on male fathead minnows and pronounced effects on carp. *JAWRA Journal of the American Water Resources Association*, *50*.

Nitrogen loading. (n.d.). Retrieved April 28, 2019, from http://greatlakesmapping.org/great_lake_stressors/7/nitrogen-loading

Pal, A., Gin, K. Y., Lin, A. Y., & Reinhard, M. (2010). Science of the total environment impacts of emerging organic contaminants on freshwater resources: Review of recent

occurrences, sources, fate and effects. *Science of the Total Environment*, *408*(24), 6062-6069. https://doi.org/10.1016/j.scitotenv.2010.09.026

Palace, V. P., Evans, R. E., Wautier, K. G., Mills, K. H., Blanchfield, P. J., Park, B. J., … & Kidd, K. A. (2009). Interspecies differences in biochemical, histopathological, and population responses in four wild fish species exposed to ethynylestradiol added to a whole lake. *Canadian Journal of Fisheries and Aquatic Sciences*, *66*(11), 1920-1935. https://doi.org/10.1139/F09-125

Park, W., Belova, E., Fu, G. Y., & Tang, X. Z. (1999). Plasma simulation studies using multilevel physics models. *Physics of Plasma, 6*(5).

Parrott, J. L., Wood, C. S., Boutot, P., & Dunn, S. (2003). Changes in growth and secondary sex characteristics of fathead minnows exposed to bleached sulfite mill effluent. *Environmental Toxicology and Chemistry*, *22*, 2908-2915.

Parrott, J. L., & Wood, C. S. (2002). Fathead minnow lifecycle tests for detection of endocrine-disrupting substances in effluents. *Water Quality Research Journal of Canada*, *37*(3), 651-667.

Pellegrini, A. F. A., Wisenden, B. D., & Sorenson, P. W. (2010). Bold minnows consistently approach danger in the field and lab in response to either chemical or visual indicators of predation risk. *Behavioral Ecology and Sociobiology*, *64*, 381-387. https://doi.org/10.1007/s00265-009-0854-y

Peterson, B. J., Wollheim, W. M., Mulholland, P. J., Webster, J. R., & Meyer, J. L. (2001). Control of nitrogen export from watersheds by headwater streams. *Science*, *292*, 86-90.

Price, P. W. (1972). Parasitoids utilizing the same host: Adaptive nature of differences in size and form. *Ecology*, *53*(1), 190-195.

Purdom, C. E., Hardiman, P. A., Bye, V. V. J., Eno, N. C., Tyler, C. R., & Sumpter, J. P. (1994). Estrogenic effects of effluents from sewage treatment works. *Chemistry and Ecology*, *8*(4), 275-285. https://doi.org/10.1080/02757549408038554

Rabalais, N. N., & Nixon, S. W. (2002). Nutrient over-enrichment in coastal waters: Global patterns of cause and effect. *Estuaries, 25.*

Rearick, D. C., Ward, J., Venturelli, P., & Schoenfuss, H. (2018). Environmental oestrogens cause predation-induced population decline in a freshwater fish. *Royal Society Open Science*, *5*(10). https://doi.org/10.1098/rsos.181065

Rodvang, W., & Simpkins, S. J. (2001). Agricultural contaminants in Quaternary aquitards: A review of occurrence and fate in North America. *Hydrogeology Journal*, *9*, 44-59. https://doi.org/10.1007/s100400000114

Sargent, R. C. (1989). Allopaternal care in the fathead minnow, Pimephales promelas: stepfathers discriminate against their adopted eggs. *Behavioral Ecology and Sociobiology*, *25*(6), 379-385. https://doi.org/10.1007/BF00300183

Schäfer, R. B., Caquet, T., Siimes, K., Mueller, R., Lagadic, L., & Liess, M. (2007). Effects of pesticides on community structure and ecosystem functions in agricultural streams of three biogeographical regions in Europe. *Science of the Total Environment*, *382*(2-3), 272-285. https://doi.org/10.1016/j.scitotenv.2007.04.040

Schoenfuss, H. L., Levitt, J. T., Van der Kraak, G., & Sorenson, P. W. (2002). Ten-week exposure to treated sewage discharge has relatively minor, variable effects on reproductive behavior and sperm production in goldfish. *Environmental Toxicology and Chemistry*, *21*(10), 2185-2190.

Scott, G. R., & Sloman, K. A. (2004). The effects of environmental pollutants on complex fish behaviour: Integrating behavioural and physiological indicators of toxicity. *Aquatic Toxicology*, *68*(4), 369-392. https://doi.org/10.1016/j.aquatox.2004.03.016

Stradling, D., & Stradling, R. (2008). Perceptions of the Burning River: Deindustrialization and Cleveland's Cuyahoga River. *Environmental History*, *13*(3), 515-535.

Tetreault, G. R., Bennett, C. J., Shires, K., Knight, B., Servos, M. R., & McMaster, M. E. (2011). Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges. *Aquatic Toxicology, 104,* 278-290.

Thomas, L. M., Jorgenson, Z. G., Brigham, M. E., Choy, S. J., Moore, J. N., Banda, J. A., … & Schoenfuss, H. L. (2017). Contaminants of emerging concern in tributaries to the Laurentian Great Lakes : II . Biological consequences of exposure. *PLoS ONE, 12*(9).

Unger, L. M. (1983). Nest defense by deceit in the fathead minnow. *Pimephales promelas, 13*(2), 125-130.

U.S. Rivers show few signs of improvement from historic nitrate increases. (2015). Retrieved April 28, 2019, from https://www.usgs.gov/news/us-rivers-show-few-signs-improvement-historic-nitrate-increases

Vitousek, P. M., Aber, J. D., Howarth, R. W., Likens, G. E., Matson, P. A., Schindler, D. W., … & Tilman, D. G. (1997). Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Application, 7*(3), 737-750.

Ward, J. L., Cox, M. K., & Schoenfuss, H. L. (2017). Thermal modulation of anthropogenic estrogen exposure on a freshwater fish, *Pimephales promelas*, at two life stages. *Hormones and Behavior, 94,* 21-32.

Wauchope, R. D. (1978). The pesticide content of surface water draining from agricultural fields-A review. *Journal of Environmental Quality*, *7*(4), 459-472.

Weber, D. N., & Spieler, R. E. (1994). Behavioral mechanisms of metal toxicity in fishes. *Molecular biological approaches to aquatic toxicology*. Boca Raton, FL: Lewis Publishers.

Wetzel, R. G. (2001). *Limnology*. New York: Academic Press.

Zedler, J. B. (2003). Wetlands at your service: Reducing impacts of agriculture at the watershed scale. *Front Ecol Environ, 1,* 65-72.

Appendix A: SOP Sample Collection

St. Cloud State University Aquatic Toxicology Laboratory

Water Sampling & Labeling for Analytical Chemistry SOP

Introduction and aim of the procedure

This SOP details the procedure to take and label water samples for analytical chemistry.

Supplies needed for assay:

- Sampling vials/ bottles/ containers (usually determined by analytical requirements)
- Labels
- Chain of Custody or Excel datasheet

Step 1 – General Considerations

- Water samples are taken for various reasons (embryo/ larval assay; extraction; analytical chemistry; etc.) which may require specific sampling techniques (i.e., larval assays)
- When water samples are taken for analytical chemistry, the overriding instructions are those provided by the analytical lab (for example, USGS Water Quality Lab Denver; Wooster College; AXYS Analytical, Canada) and should always be followed first. However, those instructions can be further augmented by the considerations below.
- The nature of the research of the Aquatic Toxicology Laboratory – using minute quantities of pollutants – requires extreme attention to cross-contamination hazards. Always (!) wear gloves; always start with control samples; always cap sampling containers quickly; always avoid any chance for cross-contamination

Step 2 – Labeling

- Every sample needs to be labeled. Any label needs to include (i) identification of the Aquatic Tox Lab, (ii) a unique label code, (iii) date, and (iv) treatment code. Additional

information may include: (a) approximate sample volume; (b) nominal concentration of the chemical(s) in the sample, (c) duplicate sample, (d) other information either requested by the analytical chemistry lab or considered helpful for later purposes (i.e., weather conditions).

- Unique Label Code – this is NOT the same as the treatment name and date (Mix-Low 8.8.18 – is a treatment name that could be on the sampling container but is NOT a unique label). Here are the requirements for unique label codes:
    - The same number of characters for ALL samples (for example: three-digit number, dash, three-letter code 111-aaa)
    - Brief to be easy to write, long enough to be unique for all samples
    - Code avoids any chance for confusion by separating letters from numbers and by underling the entire code
    - → MAKE SURE THE CODE IS DESCRIBED IN YOUR LAB NOTEBOOK

Step 3 – Sampling Considerations

- ALWAYS sample all treatments including carrier control and blank control
- ALWAYS sample in duplicate
- ALWAYS rinse bottle at least three times downstream before collection
- Store sample and duplicate samples in different places (i.e., separate freezers) if possible.
- Apply label before you sample
- Always start with least contaminated sample and work up to highest concentrations (for example: blank > ethanol control > low > medium > high)
- Do everything to avoid cross-contamination – realize that your body is the most likely source for cross-contamination!

- Cap sample containers as soon as they are full
- Store sample containers appropriately (i.e., fridge/ freezer) as soon as possible
- Maintain a data sheet of all samples

Step 4 – Storage Considerations

- If possible, store duplicate samples away from the main sample
- If the cap was left loose for freezing, tighten as soon as a sample is solidly frozen
- Ship samples to the analytical lab as soon as possible (keep back duplicate sample)
- Make sure to fill out chain-of-custody forms and keep good records.

Appendix B: SOP C-Start Assay

St. Cloud State University Aquatic Toxicology Laboratory

Fathead Minnow Laboratory Boldness Assay SOP

Introduction and goal of procedure:

This SOP details the assessment of how willing male and female FHM are to examine a novel object in their tank.

Necessary Supplies:

- Novel Object (i.e., blue die with magnet
- GoPro
- MP4 Player
- Stopwatch
- Data Sheet

Test procedure

1. Position GoPro above the tank, front one-third. Make sure there is a standard floor grid with concentric rings for this test under the tank.
2. To start of a test, slowly guide the object (i.e., blue dice) into the tank down the front wall using magnets (one the object and the other on the outside of the tank).
3. Secure the object over/on the central dot of the concentric rings of the floor grid.
4. Record the trial for 5 min with the camera. Make sure you are not visible to the fish during this time.

Scoring the tapes

1. Open up a MPEG-4 file of the video.
2. Take note of what tank number you are watching on the excel sheet

3. Latency to 1st enter outer ring (Record the time the fish first appears within the outer ring)
    a. This is when the fish's eye first crosses the line for the circle.
4. Latency to 1st enter inner ring (Record the time the fish first appears within the outer ring)
    a. This is when the fish's eye first crosses the line for the circle.
5. Record the number of times the male or female swims into the outer ring
    a. If the fish spends more than 1 second in the ring, record the amount of time it spends there (duration) to the second-millisecond decimal place (i.e., 2.32 seconds)
6. Record the number of times the male or female swims into the inner ring
    a. If the fish spends more than 1 second in the ring, record the amount of time it spends there (duration) to the second-millisecond decimal place (i.e., 2.32 seconds)
7. Total duration spent in the inner ring.
    a. Take what you found in 6 and add them all together
8. Record how many times the male or female fish bumps its nose on the die.
9. Record how many times the male or female fish charges at the die.
    a. The fish will swim fast toward the die and run into it.
10. Do this for 5 minutes of the video. If the video is longer, only score the first 5 minutes, the GoPro is in the tank.

Appendix C: SOP Feeding Assay

St. Cloud State University Aquatic Toxicology Laboratory

Fathead Minnow Laboratory Courtship Assay SOP

Introduction and goal of procedure:

This SOP details the assessment analysis of sexual preference by a "resident" male FHM when exposed to a gravid female.

Necessary Supplies:

- Gravid female FHM
- Glass bowls
- Mesh
- Rubber bands
- GoPro
- MP4 player
- Stopwatch
- Datasheet

Test procedure

1. One day prior to test day, remove all females from tanks.
2. Gather 8 different females to be tested and label each one 1-8.
3. Position GoPro above the tank, just a little closer to the front of the tank than halfway. Make sure there is a standard floor grid with concentric rings for this test under the tank.
4. Place a female in a glass bowl filled with water and secure a piece of netting/tulle over the top with an elastic. Make sure there is not a lot of excess material.

5. Tilt the bowl on an angle (to prevent air bubbles) and slide it into the tank. Once in, turn it upside down (so netting is on bottom) and position it in the center of the inner floor circle.
6. Record the trial for 5 min with the camera. Make sure you are not visible to the fish during this time.

Scoring the tapes

1. Open up a MPEG-4 file of the video.
2. Take note of what tank number you are watching on the excel sheet
3. Latency to 1st outer ring (Record the time the fish first appears within the outer ring)
    a. This is when the fish's eye first crosses the line for the circle.
4. Number of approaches by the resident male
5. Number of nest lead attempts
    a. Male swims up to the female and quickly back to nest site
6. Number of broad side displays
7. Number of bumps on glass
8. Total Time spent interacting with glass.
9. Total time spent within 2 cm of the glass
10. Do this for 5 minutes of the video. If the video is longer, only score the first 5 minutes, the GoPro is in the tank.

Appendix D: SOP Boldness Assay

St. Cloud State University Aquatic Toxicology Laboratory

Fathead Minnow Laboratory Nest Defense Assay SOP

Introduction and goal of procedure:

This SOP details the assessment analysis of nest defense by a "resident" male FHM when exposed to an "intruder" male FHM introduced into the aquarium in a glass vessel and analysis of videos.

Necessary Supplies:

- Glass bowl
- Mesh netting
- Rubber bands
- GoPro
- MP4 player
- Stopwatch
- Datasheet

Test procedure

1. Make sure there is a standard floor grid with concentric rings for this test under the tank.
2. Gather 8 different males to be tested as "intruder" males and label each one 1-8.
3. Place "intruder" male into a glass bowl with water taking note of which number he is.
4. Cover the bowl with mesh netting secured with a rubber band.

   a. Trim the edges as close to the edge as possible (this allows the “resident” male to see the “intruder” clearly.

5. Place the glass bowl into the test tank mesh side down in the center of the target on the grid.
6. Record with a GoPro for 5 mins, making sure the tank number is visible and said before placing the GoPro on the side of the tank.
7. Use each male for 4 trials.
8. Repeat steps 2-6 for the entire assay.

Scoring the tapes

1. Open up a MPEG-4 file of the video.
2. Take note of what tank number you are watching on the excel sheet
3. Latency to 1st enter outer ring (Record the time the fish first appears within 3 cm of the mirror)
   a. Note, each square is 1cmx1cm.
4. Number of times the fish approaches within 3cm of the mirror.
5. Total duration of time the fish spends within 3cm of the mirror
6. Number of broadside (lateral) displays
   a. The male will be parallel to the mirror to show his side.
7. Latency to 1st mirror bump with the snout.
8. Number of times the fish bumps the mirror.
9. Number of times the fish bites the mirror. (This might be hard to do, but
10. Duration of interaction bouts with the mirror
    a. Length of time the male continuously touches the mirror with his snout

b. This is not needed for single bites/bumps

11. Do this for 5 minutes of the video. If the video is longer, only score the first 5 minutes, the GoPro is in the tank.

Appendix E: SOP Courtship Assay

St. Cloud State University Aquatic Toxicology Laboratory

C-start SOP

Introduction and goal of procedure:

The purpose of this SOP is to test the effects of exposure on the preditor avoidance performance of larval fathead minnows.

Necessary Supplies:

- High-Speed Camera
- External Stimulus device
- Microsoft Excel
- Image J computer software
- Videos collected of C-start response
- MS-222
- Petri dish

Procedure

1. Bring larvae to behavior analysis laboratory the day before testing to acclimate. (ensure proper light cycle and air supply)
2. The day of testing position tanks and limit unnecessary light and movement to minimize disturbance of the fish
3. Very gently transfer the larvae to the testing arena (petri dish filled with aerated well water) under high-speed camera
4. Give the fish approximately 1-2 minutes of acclimation time in the testing arena.

5. Arm the camera and stimulus device
6. Wait until the larva is positioned in the center of the arena and staying still before delivering the stimulus.
7. If no C-start was observed try again up to 3 times before declaring it a “no response”
8. Save the video
9. Repeat the process until 3 larvae from each replicate have been tested
10. After testing euthanize larvae with MS-222

Digitizing C-Start Videos

1. Open the provided excel spread sheet titled “Template for C-Start Data.”
2. Download ImageJ from http://rsbweb.nih.gov/ij/download.html
3. Open ImageJ
4. From ImageJ, open the video from the hard drive.
5. A window called “AVI Reader” will pop up- click OK
6. Video will load. If the video contains too many frames a new window will pop up saying “Out of memory.” Click OK. (Only have one video open at a time- the AVI Reader can only read so many frames in total at a time; having another video open will grossly limit how many frames you will be able to see in the next video.)
7. In the ImageJ menu window click on the box with the 5 yellow diamonds (“Point or multi-point selections”). Right-click the red triangle and specify “point tool” That box should be highlighted while you work in ImageJ
8. Use “<” and “>” to move back and forth through time in the video window.
9. Scroll forward in time until the light in the corner comes on. Click on the center of the light the precise frame the light comes on. A yellow square should show up where you clicked.

10. Hit “M” to mark that point. The new window title, “Results” should show up. It will have an area, mean, min, max, x, y, and slice along the top. If you take a point and decide that it is wrong, highlight that row in this box and delete it. To delete the point from on the video push and hold Ctrl and click on a point.
11. Push the magnifying glass button in the ImageJ menu window and put the cursor over the fish and hit the “+” sign to zoom in. After zooming, push the point selection button again.
12. Scroll forward in time (>) until the fish moves. This is usually best seen when the tip of the fish’s nose moves. This decision is subjective- sometimes, the fish jerks violently and it is easy to determine when the fish moves. Other times the fish shows a weak reaction or no reaction at all to the stimulus. If there is no reaction, scroll to the end of the video and complete steps 1-14. If the reaction is weak, then scroll to when the fish first moves. If, at first, there is a weak reaction followed by a more prominent reaction, scroll to the more prominent reaction (when the fish jerks).
13. Measure 1mm: The fish is swimming on top of a grid. Place the cursor in the corner of a square near the fish (the refraction of light through water distorts the grid, so a measurement near the fish is better). Click on the corner and a yellow square should show up where you clicked. Then hold shift and click on a corner directly to the side of it. There should now be two yellow squares labeled 1 & 2 that mark two corners of a square. Click “m”. These points should appear in the “Results” window at Points #2 and #3. In the X column, the numbers should be different. In the Y column, these two points should have the same number. If the numbers are different in the Y column, then your markers were not level. Delete these rows in the results and repeat the process.

14. Measure the length of the fish: Click on the tip of the nose of the fish. Hit “m” to record the result. Click on the tail of the fish and then click on “m” to record the result. Be careful not to click on the shadow of the fish- it is easier to scroll forward and back a few frames in order to see the tail move.
15. In the top left-hand corner of the video is the frame count. For instance, “257/391” means that you are on frame 257 out of 391. After taking the tail measurement, scroll forward 20 frames. Then click on the tip of the fish’s nose. Click “m” to record the result.
16. Scroll forward another 20 frames. Click on the tip of the nose and click “m” to record the result.
17. Scroll back to just before fish reacts. Click on the Angle tool, then click on the tip of the tail and then click on the nose of the fish. A line should appear the length of the fish. Then scroll forward until the tail passes the across the and click on the nose again. Click “m” to record the result
18. In the results window, there should now be 8 points taken:

    #1- when the light first comes on

    #2 & #3- the length of 1mm based on the grid (when fish first moves)

    #4- the tip of the fish’s nose when it first moves

    #5- the tip of the fish’s tail taken at the same time as #4

    #6- the tip of the fish’s nose after 20 frames

    #7- the tip of the fish’s nose after another 20 frames

    #8- the angle of the fish (tail-nose-nose) (wait until the tail passes the line for the second nose)

    If the video is too short or there is no reaction, then complete points #1-5.

19. Select all the data from the Results window and copy.
20. Paste this data in the excel spreadsheet under the “original raw data” tab (make sure the fish ID matches the video). The data should begin in the “Point Number” column and should end in the column labeled “Count”.
21. Clear the contents out of the Results window.
22. Repeat for each video. You should label each data set by the file name in the hard drive.
23. Mark any inconsistencies such as when the AVI Reader cannot read the file, the video is too short, etc.
24. Digitize Raw Data: In Excel, open the tab titled “Digitized Raw Data”.
    From the original Excel page, copy everything from the Treatment Fish/Point number/X/Y/Angle/Slice columns and paste it in the Digitized Raw Data page under the same headings.
25. Analysis of Data: In Excel, open the third tab titled “Analysis.”

Copy everything (Treatment Fish/Point number/X/Y/Angle/Slice) from the “Digitized Raw Data” tab and paste it in column J-Q. The data will then be transferred to the appropriate columns A-H.

Make sure to enter the Treatment/Replicate (#)/Trial ID (A, B, C).

example (5.9.19_BIR_IN_1_A) Treatment=BIR_IN, Replicate=1, Trial=A

For the videos that had issues (i.e. no reactions, false starts, a video could not be opened) list those in the appropriate rows.

Highlight videos that have a latency of less than 10. Those might be false starts and should be noted.

Appendix F: SOP Nest Defense Assay

St. Cloud State University Aquatic Toxicology Laboratory

Fathead Minnow Larval Feeding Assay SOP

Introduction and goal of procedure:

The purpose of this SOP is to test the effects of any given water sample on the feeding efficiency of larval fathead minnows.

Necessary Supplies:

- Recently hatched live brine shrimp
- Larval (21 days old) fathead minnows exposed to sample water
- Dissecting microscope
- 6-well VWR sterile culture plate (~10mL volume wells)
- Pipette
- Microscope slides
- Stopwatch
- MS-222

Procedure

1. Two days before the assay

    Start brine shrimp eggs (1 tsp salt, 1 tsp frozen eggs, 1 liter well water. Aerate in 1 liter Erlenmeyer flask)

2. The day before the assay

    1. Bring larvae to behavior analysis laboratory (ensure proper light cycle)

    2. Fill wells of VWR plate with 8ml of treatment water (3 wells per replicate)

3. Carefully transfer one larva to each well (3 larvae per replicate)

4. Allow time to acclimate before the assay (overnight)

3. The day of the assay

1. Obtain live brine (approximately 150ml of shrimp from flask into separation funnel, strain/wash, and combine with ~50ml well water)

2. Pipette single drops of the shrimp mixture onto a microscope slide and count out **15±1** shrimp using a dissection microscope (record # on the data sheet)

3. Wash shrimp into well containing larva and start a **1-minute** timer

4. After 1 minute immediately euthanize larva with ms-222

5. Count remaining shrimp using a dissection microscope (record on the datasheet)

Table S1. Average exposure concentrations across three subsequent generations. Mean and standard deviation for 14 contaminants assessed in all treatments in ng/L. Sample size is provided in parenthesis. All target compounds were measured in all mixtures. Any result below the detection limit is reported as 0 ng/L.

	Contaminant	Zero Nitrate	Low Nitrate	High Nitrate
Agricultural Mixture	Atrazine	444.36±273.0(28)	392.13±289.0(28)	336.82±259.4(27)
	Bromacil	154.79±94.2(28)	134.62±80.0(28)	119.69±68.1(27)
	Metolachlor	198.78±108.0(28)	181.13±119.4(28)	144.27±95.4(27)
	Alkyl Phenols	0±0(28)	0±0(28)	0±0(27)
	Bisphenol A	71.12±30.3(28)	48.61±25.1(28)	32.06±7.91(27)
	DEET	287.50±81.0(28)	132.42±104.4(28)	243.42±89.06(27)
	Estrone	0±0(28)	0±0(28)	0±0(27)
	TBEP	2095.09±1242.3(28)	2253.12±1744.1(28)	1875.74±1236.1(27)
	Desvenlafaxine	0±0(28)	0±0(28)	0±0(27)
	Fexofenadine	0±0(28)	0±0(28)	0±0(27)
	HHCB	0±0(28)	0±0(28)	0±0(27)
	Metformin	0±0(28)	0±0(28)	0±0(27)
	MHBT	0±0(28)	0±0(28)	0±0(27)
	Sulfamethoxazole	0±0(28)	0±0(28)	0±0(27)
EtOH Mixture	Atrazine	0±0(30)	0±0(26)	0±0(26)
	Bromacil	0±0(30)	0±0(26)	0±0(26)
	Metolachlor	0±0(30)	0±0(26)	0±0(26)
	Alkyl Phenols	0±0(30)	0±0(26)	0±0(26)
	Bisphenol A	0±0(30)	0±0(26)	0±0(26)
	DEET	0±0(30)	0±0(26)	0±0(26)
	Estrone	0±0(30)	0±0(26)	0±0(26)
	TBEP	0±0(30)	0±0(26)	0±0(26)
	Desvenlafaxine	0±0(30)	0±0(26)	0±0(26)
	Fexofenadine	0±0(30)	0±0(26)	0±0(26)
	HHCB	0±0(30)	0±0(26)	0±0(26)
	Metformin	0±0(30)	0±0(26)	0±0(26)
	MHBT	0±0(30)	0±0(26)	0±0(26)
	Sulfamethoxazole	0±0(30)	0±0(26)	0±0(26)
Urban Mixture	Atrazine	0±0(28)	0±0(28)	0±0(28)
	Bromacil	0±0(28)	0±0(28)	0±0(28)
	Metolachlor	0±0(28)	0±0(28)	0±0(28)
	Alkyl Phenols	2579.39±1393.0(28)	1711.32±1011.7(28)	3088.43±1918.0(28)
	Bisphenol A	3619.32±3151.7(28)	3982.95±3407.3(28)	4902.87±3669.4(28)
	DEET	2955.60±2456.8(28)	2908.34±2468.2(28)	3723.96±2916.8(28)
	Estrone	0±0(28)	0±0(28)	0±0(28)
	TBEP	18.233.37±16751.3(28)	17636.88±16076.5(28)	21710.33±17759.1(28)
	Desvenlafaxine	1969.05±1435.6(28)	1291.52±991.2(28)	1460.84±727.4(28)
	Fexofenadine	2317.28±1857.2(28)	2167.07±1658.3(28)	2888.87±1756.02(28)
	HHCB	864.89±346.4(28)	598.27±302.4(28)	1105.37±602.38(28)
	Metformin	2207.12±1719.9(28)	2373.32±1712.7(28)	2718.12±1727.4(28)
	MHBT	10942.09±9377.13(28)	9575.49±7992.4(28)	12865.29±9900.3(28)
	Sulfamethoxazole	472.45±393.0(28)	528.52±432.727(28)	687.52±494.332(28)