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Complex Agricultural Mixtures: Assessing Effects on Aquatic Species (Pimephales promelas and Lepomis spp.) through Short-Term Field and Multi-Generational Laboratory Exposures

Nicholas Cipoletti
St. Cloud State University, ncipoletti@gmail.com

Complex Agricultural Mixtures: Assessing Effects on Aquatic Species (*Pimephales promelas* and *Lepomis* spp.) through Short-Term Field and Multi-Generational Laboratory Exposures

by

Nicholas Cipoletti

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Thesis Committee:
Heiko Schoenfuss, Chairperson
Satomi Kohno
Nathan Bruender
Mark Brigham

Abstract

In the aquatic environment, organisms are exposed to complex chemical mixtures throughout life, producing effects not anticipated in laboratory settings designed to test acute exposures of single chemicals. Exposure to chemical mixtures often produces results either not observed or counterintuitive to single chemical exposures. By employing field and laboratory-based exposures using fathead minnows and sunfish, exposures included sensitive life stages otherwise unobserved in adult acute exposure experiments. Field-based studies exposed fathead minnows and sunfish to water collected from sites along the Maumee River (Toledo, OH) to determine the impacts of a land use gradient (upstream – agriculture to downstream – industry and urban). Adult minnows were analyzed for fecundity, physiology, and hematological characteristics (vitellogenin, glucose, 11-keto testosterone, estradiol). Larval minnows were analyzed for growth, predator-avoidance behavior, feeding efficiency, and survival. Embryonic minnows were analyzed for viability, deformities, and time to hatch. Sunfish were either deployed at river sites or resident sunfish were collected for physiology, hematological characteristics (vitellogenin, glucose), and histological analysis. Results of these experiments demonstrate the effect of changing land use on aquatic organisms including reductions and delays in fecundity, alterations to metabolic indices, and greater severity of biological responses of fish exposed to waters from urban settings. Laboratory exposures analyzed the effects of eight co-occurring chemicals found in the Great Lakes watershed in areas of agricultural land use. Fathead minnows were exposed to a complex mixture of environmentally measured concentrations over three generations in a flow-through exposure system to assess the potential physiological, organism, and population level effects. Adult minnows were analyzed for physiology, hematological characteristics (vitellogenin, glucose), behavior, and fecundity. Larval fish were analyzed for growth, predator-avoidance behavior, and feeding efficiency. Juvenile fish were analyzed for growth. Adult minnows demonstrated reductions in fecundity at environmental concentrations in the second exposure generation. In addition, adults demonstrated increasing plasma vitellogenin and glucose, highlighting potential improper direction of energy. Larval and juvenile minnows were unaffected by mixture exposure. The alterations in both field and laboratory settings indicate the extent to which environmentally relevant agricultural mixtures have the potential to pose threats to aquatic organisms through reductions in fecundity, alterations to plasma proteins, and changes in physiology associated with contaminant exposure. Further, the use of a laboratory study demonstrates the need for complete life-cycle assessment of contaminants as indicated by differences between first and second-generation responses. Agricultural practices, and associated aquatic pollution, pose a threat to both the organism and population level of two North American species.

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[1] St. Cloud State University, Aquatic Toxicology Laboratory, St. Cloud, MN

[2] US Fish & Wildlife Service, Columbus, OH

[3] Southern Illinois University, Carbondale, IL

[4] USGS National Water Quality Laboratory, Denver CO

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Chapter 1. Literature Review

1.1 Introduction

The transition from a hunter-gatherer society to one dependent on agricultural practices allowed for the concentration and growth of human populations (Vasey, 1992). While this shift to a less nomadic life style meant greater food security, it also resulted in exploitation of land used for agricultural purposes (Blaikie & Brookfield, 1987). Agriculture, and the stability which it brought to developing civilizations, continued to develop over centuries to the high-intensity form which currently exists in the 21st century (Vasey, 1992). Despite the benefits associated with advances in agriculture (Evenson & Gollin, 2003), there have also been detrimental biological effects with continued conversion of natural lands for agricultural purposes including reductions in clean water and desertification (Piao et al., 2010; Schlenker & Lobell, 2010). The continued shifts to high-intensity row crop and concentrated livestock operations have resulted in aquatic pollution in surrounding bodies of water (Gómez et al., 2012; Jensen et al., 2006; Köck-Schulmeyer et al., 2012; Owens et al., 2001). It is thus necessary to understand the potential impacts of agriculturally derived contaminants on aquatic health as they pertain to the organism and population level.

1.2 Anthropogenic Alterations to Modern Agriculture

The need to feed a growing human population post World War II (circa 1940's) led to advancements in crop management and yield that were a direct result of shifting agricultural practices (Evenson & Gollin, 2003; Vasey, 1992). To meet necessary

demands, agriculture transitioned to the use of high-intensity tilling operations and monoculture crop production (Vasey, 1992). This transition, despite maximizing crop yield, led to environmental consequences including the need for chemical fertilizers to maintain the health of soil (Tilman et al., 2002), herbicides necessary for high-density production (Levidow, 1998), and irreversible sediment erosion caused by wind and water (Van Oost et al., 2007).

In addition to changes in crop production, there existed a need to produce inexpensive protein in high quantities as changes to the western diet shifted to greater consumption of protein (Cordain et al., 2005). This demand, coupled with the capability of producing livestock feed in monoculture settings, allowed for the development of concentrated animal feeding operations (CAFOs) in which livestock are grown in high-density operations and fed crops grown in monoculture settings (Spellman & Whiting, 2007). Like the shift in monoculture crop production, CAFOs relied heavily on livestock pharmaceuticals necessary to maintain healthy populations grown in concentrated operations (Gilchrist et al., 2007). In addition to the use of pharmaceuticals, livestock growth promoters have been used to accelerate the growth process to maximize yield and profit (Gilchrist et al., 2007; Thorne, 2007). Both livestock pharmaceuticals and growth promoters pollute aquatic ecosystems through the application of urine and feces in agricultural settings (Burkholder et al., 2007; Mallin & Cahoon, 2003).

It is through agricultural practices that aquatic environments, surrounding and downstream of agricultural operations, receive varied mixtures of contaminants dependent on the agricultural operation (Gómez et al., 2012; Richards & Baker, 1993).

1.3 Agriculture and Aquatic Environments

Agriculture has been clearly demonstrated as a major cause of aquatic pollution. The composition and concentration of agricultural-based chemicals can be directly related to land use, and certain pesticides are detected primarily within watersheds of agriculturally dominant regions (Gómez et al., 2012; Jensen et al., 2006; Köck-Schulmeyer et al., 2012; Owens et al., 2001). While each agricultural region may vary in land use and agricultural intensity, approximately 40% of the earth's land surface is currently in use to produce food for human and animal consumption (Foley et al., 2005). With such a large physical footprint, pollution occurs regardless of whether best management practices are followed or not. Agricultural erosion is known to occur during precipitation events, potentially leading to the input of contaminants of emerging concern (CEC) (Hurst & Sheahan, 2003; Kelly et al., 2015; Owens et al., 2001; Thomas et al., 2001; Wauchope, 1978; Wood et al., 2000).

While precipitation-based pollution is one of the more well documented routes of contaminant pollution, the implementation of drain tiling over recent decades has provided a direct route from field to waterbody for many pollutants (Beauchamp & Pavelis, 1987; Richard & Steenhuis, 1988). Drain tiling has been shown to account for upwards of 90% of fertilizer removal from fields (Rozemeijer et al., 2010), and the use of

drain tiles prevents natural degradation of agricultural chemicals by immediately removing soluble chemicals from soil (Sims et al., 1998). In addition to surface and drain tile routes of pollution, ground water flow also has the potential to transport agricultural contaminants from fields in which they were applied (Böhlke, 2002; Chae et al., 2004).

Although the intention of agricultural chemicals is of beneficial value, namely to increase food supplies for consumption, the downstream effects of these same chemicals necessary for production are often not taken into consideration during application. Often, agricultural contaminants in aquatic systems vary in composition, potency, and structures, leading to effects which may be exacerbated when compared to the presence of single compounds (Garcia-Reyero et al., 2009).

Agricultural derived pollution has been further exacerbated by the use of waste water treatment facilities and their relatively poor capabilities for removing pesticides, often times failing to remove any significant amount (Köck-Schulmeyer et al., 2013). Many agricultural settings apply sewage sludge as a form of fertilizer, which has demonstrated to contain detectable levels of pharmaceuticals (Jelic et al., 2011; Radjenovic et al., 2009), petroleum hydrocarbons, polyaromatic hydrocarbons, solvents such as dichloromethane and toluene, lindane (Schnaak et al., 1997), and other CECs (Diaz-Cruz et al., 2009; Harrison et al., 2006). During precipitation events, erosion of the sludge and its content adds additional pollutants to downstream aquatic environments. The combination of both agricultural chemicals in complex mixtures has not been

studied in a toxicological setting, further demonstrating the need for complex mixture exposures to agricultural CECs.

1.4 Agricultural Chemicals and their Effects on Aquatic Organisms

The single chemical exposure of aquatic organisms in a laboratory setting to agriculturally derived contaminants has resulted in known biological changes, including decreased survival and elevated cortisol stress responses (Waring & Moore, 2004), increases of the egg-yolk precursor protein in male fish (Bringolf et al., 2004), as well as feminized sex ratios (Hoskins & Boone, 2017). In addition to physiological responses, Scott and Sloman (2004) have detailed the multitude of behavioral endpoints altered due to single chemical exposure. These include reduced survival (Carlson et al., 1998; Little et al., 1990), alterations in response to alarm stimuli (Saglio & Trijasse, 1998), reductions in schooling behavior (Weis & Weis, 1974), as well as decreases in the visual recognition of predators (Wibe et al., 2001). Chemical alterations to physiological responses often lead to behavioral alterations through molecular and physiological perturbations, which in turn can affect reproduction (Scott & Sloman, 2004). Changes in courtship and spawning have been documented in the presence of 17β-estradiol (Oshima et al., 2003; Schoenfuss et al., 2002), and phenol (Colgan et al., 1982), while alterations to fecundity have been observed in the presence of ethynyl estradiol (Seki et al., 2002) and trenbolone (Jensen et al., 2006; Thrupp et al., 2018).

Field studies focused on the biological effects of agricultural CECs have demonstrated reductions in species abundance and total numbers (Schäfer et al., 2007).

While field studies demonstrate true ecosystem wide alterations, they fail to identify the effects solely associated with agricultural CEC exposure and are limited in their capability of observing behavioral and reproductive changes. Combining the environmentally observed chemical mixtures (Elliott et al., 2017) with the control of a laboratory setting better allows for observation of changes associated with chemical exposure while maintaining ecological relevance. A scarcity of agricultural chemical mixtures research exists, highlighting the necessity for further examination of agricultural chemical mixtures. Exposures utilizing fathead minnows (*Pimephales promelas*) have demonstrated alterations to predator avoidance behavior in larval fish (McGee et al., 2009) and changes in the synthesis of vitellogenin (Vtg) in exposed organisms (Brian et al., 2005; Harris et al., 2009). This lack of mixtures research, coupled with the degree of agriculturally impacted bodies of water, warrant the need for further study.

1.5 Fathead Minnow and Sunfish: Uses in Ecotoxicology

The use of a fish model is necessary to study the effects of chemical mixtures as they exist in all developmental stages (adult, larval, juvenile) in the aquatic environment. Use of fish to study the effects of contaminants is also of benefit due to the conservation of the hypothalamus-pituitary-gonadal (HPG) axis similar to that of most other vertebrates. Conservation of the HPG axis allows for the extrapolation of biological responses to vertebrate organisms (Ankley & Johnson, 2004). One commonly used model fish in toxicological research is the teleost fathead minnow (*Pimephales*

promelas) of the Cyprinidae family (Geiger et al., 1988). Fathead minnows are habitat generalists, feeding primarily on algae, aquatic invertebrates, and protozoa, and can be found throughout most of North America (Zimmer et al., 2002). Fathead minnows are highly tolerant of degraded ecosystems including poor water quality parameters such as elevated temperatures, low dissolved oxygen, high salinity concentrations, turbid environments, and variable pHs (Sommer, 2011). They range in size from 2.5-7.5 cm long at the time of sexual maturity (Paetz, 1992) with males being larger than females (Becker, 1983), and can live for 3-4 years, although predation and environmental factors limit this to approximately 2 years in wild populations (Kidd et al., 2007). Both males and females are deep-bodied with a blunt head, slightly forked caudal fin, and a single, soft rayed dorsal fin (Paetz, 1992).

Fathead minnows can continuously spawn in laboratory settings once sexual maturity is reached (Brungs, 1971; Jensen et al., 2001), making them ideal for reproduction-based laboratory studies. In natural settings, reproduction begins when waters reach 15°C and an appropriate light cycle is achieved (16:8 light:dark), typically May through August (Danylchuk & Tom, 2001; Duda, 1989; Prather, 1957). Females can produce 6,800 to 10,000 eggs in a single reproductive season (Ross, 2001). Male fathead minnows develop sexually dimorphic characteristics during the reproductive season. Sexual characteristics consist of a fatty dorsal pad, nuptial tubercles, and distinctive banding coloration (R. Smith, 1974, 1978). The success of male fathead minnow reproduction is dependent on their ability to acquire and defend a nest site, as well as

attract gravid females (Martinovic-Weigelt et al., 2012; Sommer, 2011). Defensive behaviors consist of bumping intruding males with tubercles and territorial behavioral alterations only present during the breeding season (McMillan & Smith, 1974). Dorsal pads of male fathead minnows contain fungicidal properties to disinfect eggs (Kottelat & Freyhof, 2007).

In addition to the robust reproductive effort of fathead minnows, well documented fathead minnow culture techniques, quick time to mature, accessibility in large quantities, and the ability to withstand varied conditions make the fathead minnow a common model for toxicological research (Denny, 1987; Geiger et al., 1988; Jensen et al., 2001).

The second commonly utilized species is the teleost sunfish (*Lepomis* spp.) of the Centrarchidae family (Helfman et al., 2009). Sunfish can grow in excess of 240 grams and 95 mm in length (Pflieger, 1975), with compressed bodies of various colors depending on the species (Smith, 1979). Males are often lighter in coloration than females, with bright yellow or orange breasts (Gross & Charnov, 1980). Sunfish, particularly bluegill sunfish (*Lepomis macrochirus*), are widespread throughout the Midwest and Central North America, overlapping in territory in large part with fathead minnows (Becker, 1983). Sunfish prefer clear and well vegetated waters but can be found in a variety of habitats including ponds, streams, and swamps (Becker, 1983; Smith, 1979).

Sunfish spawning begins in May when water temperatures reach 20 °C and lasts typically until August (Becker, 1983). Male sunfish, similar to fathead minnows, are responsible for attaining and maintaining a nesting site, often in gravel or sand bars of 0.3 to 0.6 meters water depth, in order to provide parental care of eggs (Becker, 1983; Gill, 1906; Gross & Charnov, 1980). Spawning occurs in colony settings with upwards of 40 to 50 nests in a 1250 m^2 littoral zone (Harlan & Speaker, 1956). During the reproductive season, males defend their nest sites from other male and female sunfish, protecting developing embryos and larvae from predation while also providing aeration of developing eggs through fanning of the pectoral and pelvic fins (Becker, 1983; Pflieger, 1975). Females spawn in multiple nests throughout the season (Pflieger, 1975) and the net average egg production of a single nest has been estimated at 4,800 eggs (Churchhill, 1976).

The ecological relevance of sunfish, in addition to well-established laboratory culture techniques, relative abundance, integral species in aquatic food webs, and widespread geographic range make the variety of sunfish species beneficial for toxicological research.

Chapter 2. Land Use Contributions to Adverse Biological Effects in a Complex and Degraded Agricultural and Urban Watershed: A Case Study of the Maumee River

2.1 Introduction

The Upper Midwest of the USA is a global leader in row crop and meat production (Hatfield, 2012). This astonishing productivity is driven by high-intensity agricultural practices include the application of fertilizer and pesticides, spreading of manure from concentrated animal feedlot operations, and drain-tiling of farmland. Unfortunately, these agricultural practices may increase chemical contamination of nearby waters and lead to the degradation and impairment of aquatic ecosystems (DeLorenzo et al., 2001; Heaney et al., 2001; Lammert & Allan, 1999). However, even watersheds associated with agricultural land uses frequently contain other pollutant sources including the presence of urban areas which may contribute wastewater effluent, urban runoff, and combined sewer overflows to an already impacted watershed. These multiple sources of anthropogenic pollution ultimately result in aquatic ecosystems exposure to complex chemical mixtures (Focazio et al., 2008; Gros et al., 2007; Pal et al., 2010; Wang, 2014). Remediation of observed adverse biological impacts in aquatic ecosystems requires an understanding of the contribution of multiple sources. Unfortunately, little is known about the contributions of multiple pollutant sources to adverse biological outcomes in large riverine systems. In the current study, the Maumee River (Ohio) in the heart of the agricultural farmland of the USA, largest

contributor to the Laurentian Great Lakes, and flowing through the metropolitan area of Toledo, OH was studied to assess whether documented biological effects were a result of cumulative chemical loads or the impact of nearby land use practices.

The Maumee River drains an area dominated by high-intensity row-crop farming and concentrated animal feedlot operations which may contribute pesticide/herbicide pollution as well as livestock specific chemicals to the receiving watershed (Burkholder et al., 2007; Gilliom, 2007; Richards & Baker, 1993). Just prior to emptying into Lake Erie, the river flows through the city of Toledo, Ohio. The presence of urban centers in a watershed often has an oversized chemical footprint and may be associated with runoff during precipitation events, resulting in episodic chemical inputs into aquatic ecosystems (Dwight et al., 2002; Taebi & Droste, 2004; Yong & Chen, 2002; Zhao et al., 2010). The Greater Toledo Area also includes a large industrial complex and harbor which may contribute contaminants to the river through runoff or lack of complete breakdown during wastewater treatment (Suthar et al., 2010). Treated wastewater effluent is discharged into the Maumee River at multiple point in the Greater Toledo Area and likely contributes a range of chemicals to the river as a result of incomplete removal during the treatment cycle (Deblonde et al., 2011; Köck-Schulmeyer et al., 2013; Rosal et al., 2010). Wastewater effluent often makes up the most consistent contribution of pollution to riverine systems regardless of precipitation, season, or land use (Lee et al., 2011). As a major river to the Great Lakes watershed and with a history of contamination, the Maumee River is an ideal system in which to study the impacts from

multiple land use practices as the land use surrounding the river transitions from agricultural to (sub-)urban, industrial, and finally wastewater effluent inputs.

The complexity of pollutants sources, chemicals mixtures, and multitude of anthropogenic stressors require a controlled experimental approach to examine the contributions of multiple pollutant sources to the documented biological impacts. Using organisms reared under controlled conditions as sentinels to assess the hazard of water sources from different regions of the river representing different land use characteristics allows for the integration of complex pollutant mixtures while excluding anthropogenic non-pollutant confounding variables (such as degraded habitats, invasive species, human activity). Using river-side exposure infrastructure, in contrast to assessing caged or resident animals, also allows for greater control of physicochemical variables (i.e., pH, temperature, dissolved oxygen) that may affect biological organisms beyond the presence of chemical mixtures. The use of mobile exposure laboratory platforms for such experiments has been advocated previously (Kolok et al., 2012; Minarik et al., 2014) and was employed in the current study. By exposing fish under controlled conditions to whole water samples from stream reaches associated with agricultural, urban, industrial, effluent-dominated land use, the contribution of each of the complex chemical mixtures to fish health could be assessed. It was, therefore, expected that agricultural land would be associated with the presence of pesticides, herbicides (Elliott et al., 2017; Nowell et al., 2018), and livestock specific chemicals (ie. growth promoters, steroids, livestock pharmaceuticals) (Jaffrézic et al., 2017). Biological effects from

agricultural pollutants include feminized sex ratios (Hoskins & Boone, 2017), reductions in fecundity (Jensen et al., 2006), and a reduction in species abundance and total number (Schäfer et al., 2007). In contrast, we expected urban areas to contribute contaminants including heavy metals, nutrients, and suspended solids (Brezonik & Stadelmann, 2002), as well as polycyclic aromatic hydrocarbons (Van Metre et al., 2000). Potential biological effects include acute toxicity in invertebrate species (Bay et al., 2003), and pre-spawning mortality among mature fish (Scholz et al., 2011). The presence of industrial facilities may lead to aquatic pollutants including pharmaceuticals (Larsson et al., 2007), surfactants (Field & Reed, 1996), and synthetic sterols (Desbrow et al., 1998). These industrial derived pollutants often result in biological effects in aquatic organisms such as male-biased sex ratios (Larsson & Förlin, 2002), alterations in reproductive physiology (Van den Heuvel & Ellis, 2002), and increases in plasma vitellogenin concentrations indicative of estrogenic consistency (Tremblay & Van Der Kraak, 1999). Lastly, wastewater effluent contain pollutants ranging from pharmaceuticals and personal care products, to insecticides, musks, fragrances, and a variety of other contaminants of emerging concern (Zhang et al., 2017). The biological effects of such a diversity of chemicals may include changes in reproductive output (Thrupp et al., 2018), alterations to embryonic development (Zhang et al., 2017), and larval survival (Rearick et al., 2014). Adult fish may experience suppression of male reproductive behaviors (Martinovic et al., 2007) and changes in plasma sex hormone concentrations (Hemming et al., 2001; Jobling et al., 2004).

The overarching objective of this study was to assess the biological impacts of an agricultural and urban degraded river system at seven field sites selected based on a variety of land uses and potential pollutant input characteristics (Table 2.1) on multiple life stages of the fathead minnow. We chose this species as it is native to most of North America, an important component of the aquatic food chain, and commonly used in toxicological exposure experiments. Mature male and female fathead minnows (*Pimephales promelas*) were maintained in a mobile exposure laboratory trailer and exposed to water samples spanning an agricultural to urban, upstream to downstream, gradient. Larval fathead minnows were exposed in a laboratory setting to aide in understanding the impacts of a degraded river system on the sensitive developmental stages. Embryonic exposures were conducted to determine potential developmental abnormalities associated with pre-hatching development. Use of the fathead minnow at three varying life stages allowed for the interpretation of potential reproductive and physiological (adult), behavioral (larval), and developmental (embryo) alterations. When combined, this multi-faceted data set provides a comprehensive assessment of the contribution to biological impacts of a variety of land-use-dependent chemical mixtures on fish health.

Table 2.1. Field site characteristics of Maumee River field collection sites listed from upstream (GRM) to downstream (TWP). Characteristics include site name (abbreviation), watershed basin (km^2), upstream population as of 2010 census, land use cover within watershed (%), latitude/longitude coordinates of sample collection site, the distance to the next downstream site (km), and the distance to the river mouth (km).

Site (Abbreviation)	Basin (km^2)	Population (2010)	Land Use (%)							Latitude	Longitude	Km to next downstream site (km to river mouth)
			Urban High	Urban Medium	Urban Low	Forest	Grass	Crop	Water			
Grand Rapids Marina (GRM)	61	2,153	0.1	0.7	2.6	8	1.3	74	5.3	-83.88694	41.41251	NA (52)
Beaver Creek (BCR)	43	756	0.2	0.1	0.6	6.3	0.9	82.3	0.1	-83.84465	41.39365	NA (54)
Farnsworth Metro Park (FMP)	41	7,614	0.3	2.1	9.8	5.1	1.6	64.1	3.5	-83.74889	41.47661	15 (38)
Perrysburgh (PBG)	49	16,235	2.3	5.6	25.6	9.1	1.2	17.5	13.4	-83.63233	41.56108	15 (22)
Upstream of Swan Creek (USC)	43	46,715	10.4	24.3	34	0.7	0.5	0.5	16.5	-83.53139	41.63697	13 (9)
Swan Creek (SCR)	95	62,564	6.6	14.2	23.9	7.7	0.8	31.1	0.1	-83.54285	41.64476	NA (9)
Toledo Water Reclamation Plant (TWP)	43	46,715	10.4	24.3	34	0.7	0.5	0.5	16.5.	-83.47557	41.69061	9 (1)

2.2 Materials & Methods

2.2.1 Study River and Study Sites

The Maumee River used for this case study is a historically degraded watershed as a result of agricultural practices and urban development (Slocum, 1905). The Maumee River forms in Fort Wayne, IN, as the confluence of the St. Joseph and St. Mary's rivers before flowing 220 km through northwest Ohio and discharging into Lake Erie. The Maumee River watershed is characterized by glacial moraine influenced soils. Prior to human settlement its watershed was composed of wetlands, forests, and grasslands forming "The Great Black Swamp" – the last region of the southern Laurentian Great Lakes to be colonized by European settlers (Mollenkopf, 1999). Converting the Great Black Swamp into productive farmland required extensive drain

tiling and the channelization of the Maumee River during the 19th century. Today approximately 80% of the watershed is under agricultural use, primarily dedicated to corn and soybean production while urban land cover accounts for 11% of the Maumee River watershed (*Evaluation of Land Use / Land Cover Characteristics in Ohio Drainages to Lake Erie*, 2008). The Maumee River has become heavily polluted with sediment and phosphorus partially due to the altered soil characteristics from agricultural improvements and the draining of the former swamp (Baker et al., 2014; Stow et al., 2015). As a consequence, the river contributes frequently to algal blooms in the western portion of Lake Erie (Michalak et al., 2013). The extensive agricultural use coupled with an abrupt shift to a large urban population in Toledo, OH, makes the watershed an ideal model to examine the role of an agriculturally degraded and urban influenced river.

Seven study sites were selected to evaluate a gradient of land uses common in the Great Lakes Basin, starting with more agricultural influences upstream and increasing urban influence downstream (Figure 2.1). Beaver Creek (BCR) is a small side-stream that drains poultry and dairy farmland before it empties into the Maumee River just downstream of Grand Rapids, OH, USA. Grand Rapids (GRM) was the most upstream site on the main stem of the Maumee River, located immediately above a dam near the town of Grand Rapids, OH. Further downstream, the Farnsworth Metro Park site (FMP) was the third and final site with substantial nearby agricultural land use, and was located immediately upstream of Waterville, OH. Perrysburg (PBG) represents the

transition from agricultural to sub-urban land use and receives treated wastewater effluent from this small community. A site just upstream of Swan Creek (USC) was chosen to delineate the transition from suburban to urban and industrial land use. Swan Creek (SCR) is a small side-stream that flows mostly through residential suburbs of Toledo before emptying into the Maumee River in downtown Toledo, OH. Swan Creek also contains multiple combined sewer overflows (CSOs), wastewater treatment plants (WWTPs), and storm water runoff discharge pipes from downtown Toledo, OH. The most downstream site, just below the Toledo wastewater treatment plant (TWP), was located less than 2km from the confluence with Lake Erie.

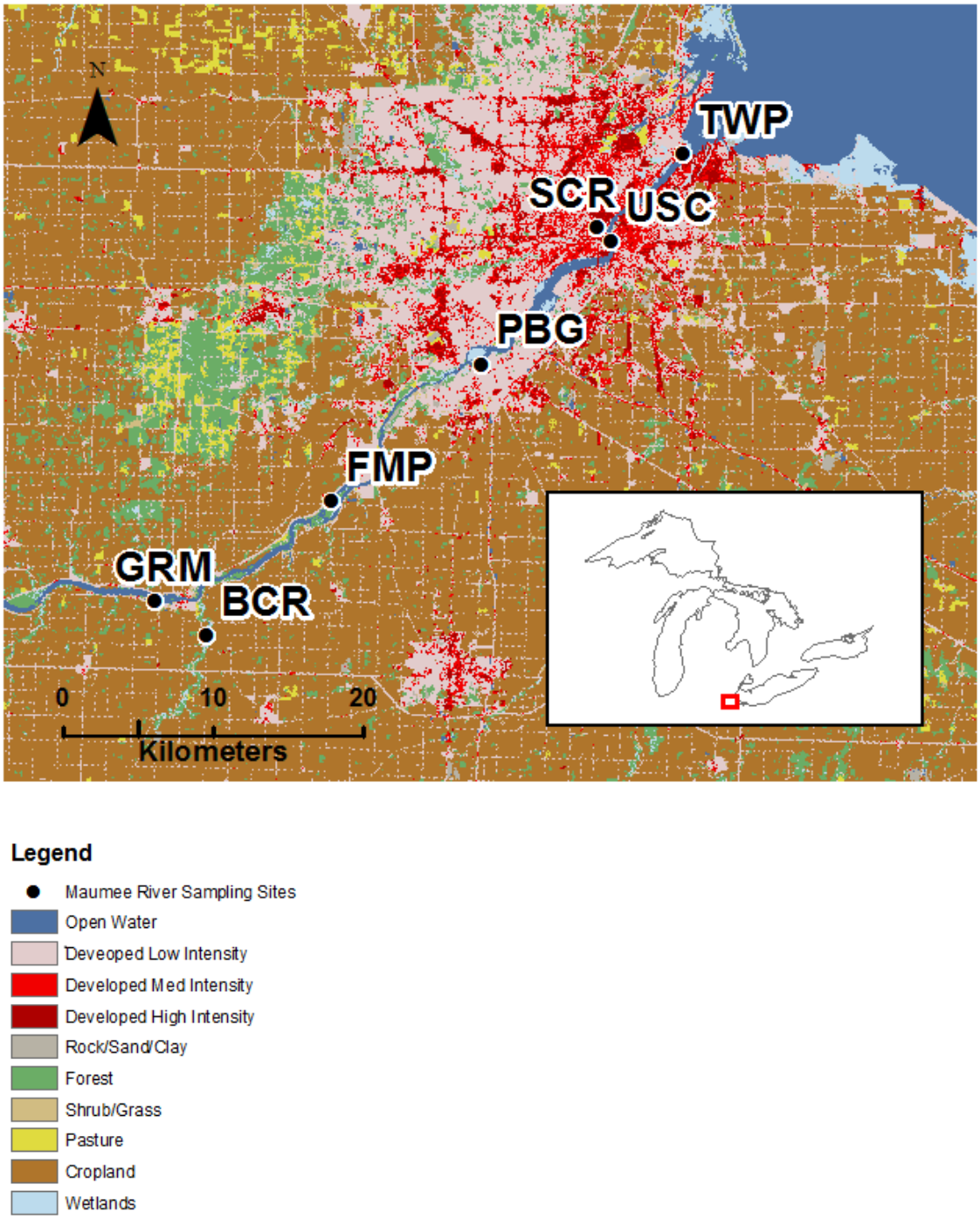

Figure 2.1. Maumee River study site locations, refer to Table 2.1 for site abbreviation codes. River flow from GRM (upstream) to TWP (downstream). Increasing urbanization represents Toledo, OH.

2.2.2 Surface Water Chemical Analysis

In addition to water samples collected for the daily 50% aquarium water renewal, surface water samples, along with negative and positive controls, were collected once per week during the 21-day fish exposure from each site for chemical analysis (n=4/site). Briefly, at the time of water collection for the fish exposures, 2L of water were collected in High Density Polyethylene (HDPE) bottles and 3L were collected in baked amber glass bottles. All bottles were conditioned with site water prior to sample collection. Samples were cooled and shipped on ice to AXYS Laboratories (Sidney, British Columbia) and Southern Illinois University (Carbondale, Illinois) for chemical analysis.

At AXYS Laboratories, samples were analyzed for a suite of multi-residue pesticides (MREs), hormones, and pharmaceuticals and personal care products (PPCPs). Multi-residue pesticides (AXYS Method MLA-035) were analyzed using high resolution gas chromatography/high resolution mass spectrometry (HRGC/MS) following methods described in EPA Method 1699. Hormones and PPCPs (AXYS Method MLA-075) were analyzed using high performance liquid chromatograph reversed phase C18 or HILIC column, coupled to a triple quadrupole mass spectrometer (LC-MS/MS), modified from methods described in EPA Method 1694.

2.2.3 Chemical Data Treatment

Following methods described in Elliott et al. (2017) and Thomas et al. (2017), chemical results were reduced to one sample per site per year. This reduction was

achieved by using the maximum concentration detected for each chemical at every site for both 2016 and 2017. Maximum concentrations were used for this study due to the abundance of left-centered data (i.e., non-detects), and due to the limited number of samples per site (n=4/site) that would result in insufficient data for other estimation techniques (e.g., maximum likelihood). Additionally, by using the maximum concentration for a given chemical, that concentration will signify the greatest toxicological concern for fish exposed in this study, which is relevant when evaluating biological endpoints. However, due to the limited sampling campaign, the maximum concentration of any detected contaminant may have been greater than the sampling effort indicates. To provide further interpretive ability when evaluating chemical input and comparing to biological endpoints, chemicals were categorized into four chemical classes based on their commercial use; Hormones, Multi-Use, Pesticides, and Pharmaceuticals. Total chemical concentrations were calculated for each site; first by total chemical concentration for each class for each site, and then total chemical load for each site.

2.2.4 Exposures

2.2.4.1 Adult Exposure

21-day fathead minnow exposures were conducted between May/June 2016 and May 2017. All fish exposure protocols were approved by the St. Cloud State University Institutional Animal Care and Use Committee (IACUC permit #8-82; 8-107). We applied a daily 50% static renewal procedure using waters collected from each field site to

minimize sample degradation in the exposure aquaria. 12L collection containers were cleaned using filtered city water (filtered using two carbon filters; Hydronix, Chino Hills, CA) between sampling events. In addition, a negative (BLK) and positive control (MIX) were included in the experimental design. The BLK control consisted of filtered city water. The MIX control consisted of filtered city water with a spike of co-occurring agricultural chemicals (Table 2.2).

Table 2.2. Nominal concentrations of positive control (MIX) used in Maumee fathead minnow 21-day exposure. Chemicals used are common co-occurring agricultural derived chemicals, listed as ng/L.

Chemical	Concentration (ng/L)
Metolachlor	1700
Atrazine	4000
DEET	2000
TBEP	21000
Bromacil	1200
Estrone	240
BPA	600
Alkyl Phenols	1880

Fathead minnow exposure occurred in a mobile exposure laboratory trailer (Figure 2.2), previously employed in field applications (Minarik et al., 2014) and easy to maneuver/accommodate to field exposures (Kolok et al., 2012). Fathead minnow exposure conditions followed recommended USEPA guidelines (Denny, 1987), including consistent temperature (2016 - 21.4 °C ± 1.3; 2017 – 22.3 °C ± 2.0), dissolved oxygen (>5mg/L), and photoperiod (16 L:8 D). The only alteration to USEPA guidelines

was a change in exposure temperature, from recommended 25 °C to 21 – 23 °C, to approximate ambient temperatures in the Maumee River. Mature male and female fathead minnows were shipped overnight from Environmental Consulting & Testing (Superior, WI) and randomly assigned to one of 180 3L exposure aquaria. Each aquarium contained one male and one female fish, one inverted semi-circle PVC tile for spawning, and an air stone to maintain dissolved oxygen concentrations above 5mg/L. Each treatment consisted of 20 experimental replicates (aquaria). Water quality parameters were checked daily using a YSI (556 MPS, YSI Incorporated, Yellow Springs, OH) to record temperature (°C), conductivity (µs/cm), total dissolved solids (g/L), salinity, dissolved oxygen (mg/L), pH, and oxidation reduction potential. Daily 50% static renewals were performed for all aquaria by siphoning out 1.5L of water before refilling with appropriate treatment water using a pitcher pot (Supplemental SOP 2.1). Fish were fed twice daily *ad libitum* with a pre-mixed solution of 2:1 frozen brine shrimp:blood worms (Brine Shrimp Direct, Ogden, UT) dissolved in filtered city water. Aquaria were checked daily for reproduction (fecundity as mean number of eggs per female per treatment) and mortality.

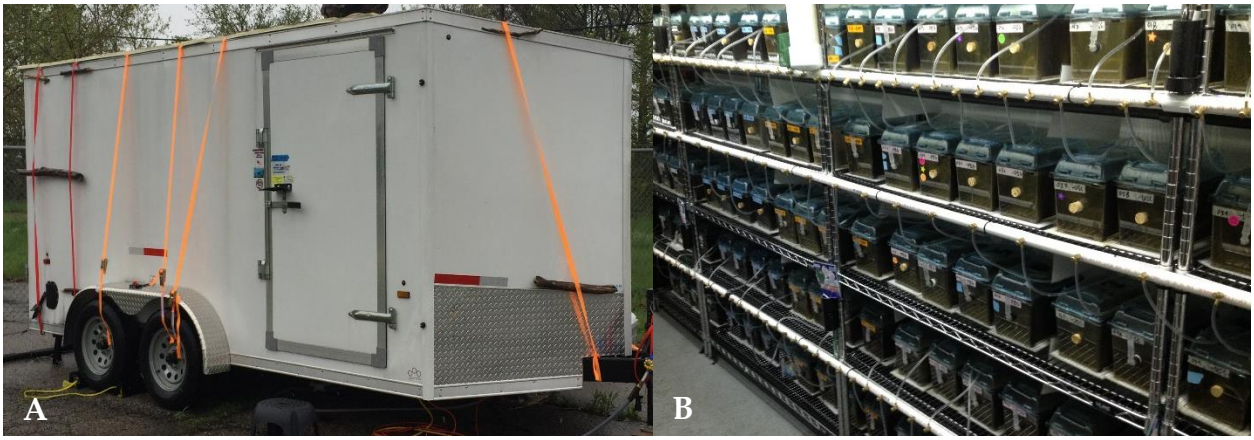

Figure 2.2. Mobile exposure laboratory trailer (MELT) (A) and internal mobile exposure laboratory trailer exposure aquaria (B) used for 21-day adult fathead minnow exposures.

2.2.4.2 Larval Exposure

Larval exposures were conducted at the Aquatic Toxicology Laboratory at St. Cloud State University (St. Cloud, MN, USA) using 30L grab water samples collected during the adult fathead minnow exposure, returned overnight to St. Cloud and frozen (-20C) until later use. Post-hatch fathead minnow larvae (<24 hours old) were shipped from Environmental Consulting & Testing (Superior, WI) overnight to St. Cloud. For each treatment, larvae were randomly placed in 1L glass jars (n=20 per jar, 2016 - 4 jars per treatment; 2017 – 6 jars per treatment) and exposed to site-specific water samples. Larvae were maintained at environmental temperatures (22.71 ± 1.43 °C) and photoperiod (16 L:8 D) within a laminar flow hood to reduce mold growth. Larvae received a 50% static daily water exchange of grab samples collected on the final day of water sampling for each respective MELT exposure (coinciding with surface water sampling for chemical analysis by AXYS laboratories). Larvae were fed twice daily *ad*

libitum with hatched brine shrimp (Brine Shrimp Direct, Ogden, UT). Larvae were maintained for 21 days prior to testing for predator avoidance performance, feeding efficiency, growth, and survival. Predator avoidance performance was assessed using four variables (latency, escape velocity, escape angle, and total escape response) as described previously (McGee et al., 2009).

2.2.4.3 Embryo Exposure

Eggs (<12 hours old) were collected from a breeding tile in a fathead minnow spawning pair and transferred to a 24 well plate (VWR International, Radnor, PA) pre-filled with 2mL of treatment water (2016 – n=12; 2017 – n=24). Embryos were maintained at environmental conditions of (22.71 ± 1.43 °C) and photoperiod (16 L:8 D) in a laminar flow hood. Daily 50% (by volume) static-renewal water exchanges were performed until hatching. After hatching, embryos were euthanized via an overdose of MS-222 (Argent Chemical Laboratories, Remdomn, WA) and preserved in 10% neutral buffered formalin for analysis. Developmental endpoints included a subjective rating (1 – normal development, 2 – mild abnormality, 3 – severe abnormality) of yolk sac development, spinal curvature, eye edema, cranial bulging, and swim bladder inflation (Henry et al., 1997; Lefebvre et al., 2004). Development was analyzed as the sum of developmental ratings from normal to severe (5 – 15). Embryonic development was also monitored for fertilization (%) and time to hatch.

2.2.5 Fish Biological Analysis

At the end of both 2016 and 2017 21-day fathead minnow exposures, fish were anesthetized in 0.1% MS-222 (Argent Chemical Laboratories, Remdomnd, WA) and measured for total and standard length (mm), as well as wet mass (Ohaus Scout Pro 0.1g, Parsippany, NJ). Wet weight and total length were used to calculate the condition factor [body weight (g) /total length (mm)].

To eliminate treatment bias, male fathead minnows were graded blind for the subjective expression (0-3 scale) of three secondary sexual characteristics (SSC)- dorsal pad, tubercles, and banding coloration, modified from Parrott et al. (2003) . The sum of these secondary sexual characteristics was used for statistical comparison between treatments. Plasma was collected from male and female fathead minnow by severing the caudal vasculature and collecting blood using heparinized micro-hematocrit capillary tubes (Fisher Brand, Pittsburgh, PA). Blood samples were centrifuged at 5,000 rpm for 5 minutes and separated plasma was stored at -80^{o}C until analysis. Blood glucose was recorded using a TRUEbalance blood glucose meter (Moore Medical LLC, Farmington, CT) by collecting a blood sample from the severed caudal vasculature.

Laboratory analysis of plasma samples was conducted using a competitive antibody-capture ELISA following Parks et al. (1999) for plasma vitellogenin (Vtg) quantification. Standard preparation and sample analysis followed previously described methods (Minarik et al., 2014). Male fathead minnows were also analyzed for plasma estradiol (E_2) and 11-keto testosterone (11-KT) using ELISA kits from Cayman

Chemical Company (E_2 Item 582251, Batch 0489295; 11-KT Item 582751, Batch 0489294; Ann Arbor, MI).

2.2.6 Fish Chemical Analysis

Following blood collection in 2016, fish tissues separated by sex were placed into aluminum foil. Ten fish of the same sex and from the same treatment were combined as one analytical sample (n=2). Fish were shipped on dry ice to AXYS Laboratories (Vancouver, Canada). At AXYS Laboratories, tissues were homogenized and extracted with dicholormethane via Soxhlet extraction. Samples were analyzed using HRGC/MS (AXYS Method MLA-035). For hormone and PPCP analysis, homogenates were extracted via sonication with buffered acetonitrile and with pure acetonitrile. Samples were then concentrated by rotary evaporation and diluted with ultra-pure water to 200 mL. The acid extract was then stabilized with ethylenediaminetetraacetic acid, filtered, and cleaned up by solid phase extraction. Extracts were analyzed using LC-MS/MS in positive and negative ionization modes (AXYS Method MLA-075).

Tissue chemical concentrations were evaluated following similar methods as water samples. Chemicals were categorized into four chemical classes based on their commercial use; Hormones, Multi-Use, Pesticides, and Pharmaceuticals. Samples were first separated by sex, and then total tissue concentrations were calculated for each class for each sample, followed by the total chemical load for each sample.

2.2.7 Statistical Analysis

Adult fecundity data was analyzed using a repeated measures ANOVA with a Greenhouse-Geisser correction (IBM SPSS Version 22.0). Statistical analysis for non-fecundity endpoints used one-way ANOVA followed by a Dunnett's multiple comparison post-test in which BLK was used as the single control treatment (SAS JMP Pro Version 13.2.0). Adult survival and embryonic viability were analyzed using Fisher's exact test with a two-tailed p-value. A limit of $p<0.05$ was set for statistical significance. Vtg data was log transformed for statistical analysis. E_2 and 11-KT were analyzed as the ArcSin transformed ratio: = ArcSin [E_2 / 11-KT]

2.3 Results

We conducted fathead minnow breeding pair replicate 21-day 50% daily static renewal exposure experiments in spring of 2016 and 2017. Both years, 20 breeding pairs per treatment were exposed inside a temperature and photoperiod control mobile exposure laboratory trailer to waters collected from seven sites representing an agricultural to urban gradient of the Maumee River and two of its small tributaries (agricultural Beaver Creek and residential Swan Creek). A blank filtered city water control and a positive contaminant mixture control were added to the experimental design. Both exposure experiments proceeded as planned with no major disruptions in experimental design. Weather conditions differed dramatically between a very dry 2016 (<1cm precipitation during the exposure) with low river flow conditions and a

very wet spring in 2017 (10.3cm precipitation) with localized flooding during the exposure experiments.

2.3.1 Surface Water and Tissue Chemistry

Chemicals were detected in every water (Figure 2.3) and tissue sample. A total of 37 chemicals were detected in at least half of the water samples, while 110 chemicals were detected in at least one water sample. The most commonly detected chemicals (detected in at least 82% of the samples) were (% of water samples which contained detection, chemical use): Heptachlor Epoxide (94%, pesticide), Atrazine (88%, pesticide), Benzoylecgonine (88%, multi-use), Desethylatrazine (88%, pesticide), Sulfamethoxazole (88%, pharmaceutical), Ametryn (82%, pesticide), Atenolol (82%, pharmaceutical), Caffeine (82%, multi-use), Carbamazepine (82%, pharmaceutical), Cotinine (82%, multi-use), Dieldrin (82%, pesticide), Fluoxetine (82%, pharmaceutical), Metformin (82%, pharmaceutical), Metribuzin (82%, pesticide), Naproxen (82%, pharmaceutical), Triamterene (82%, pharmaceutical), and Venlafaxine (82%, pharmaceutical).

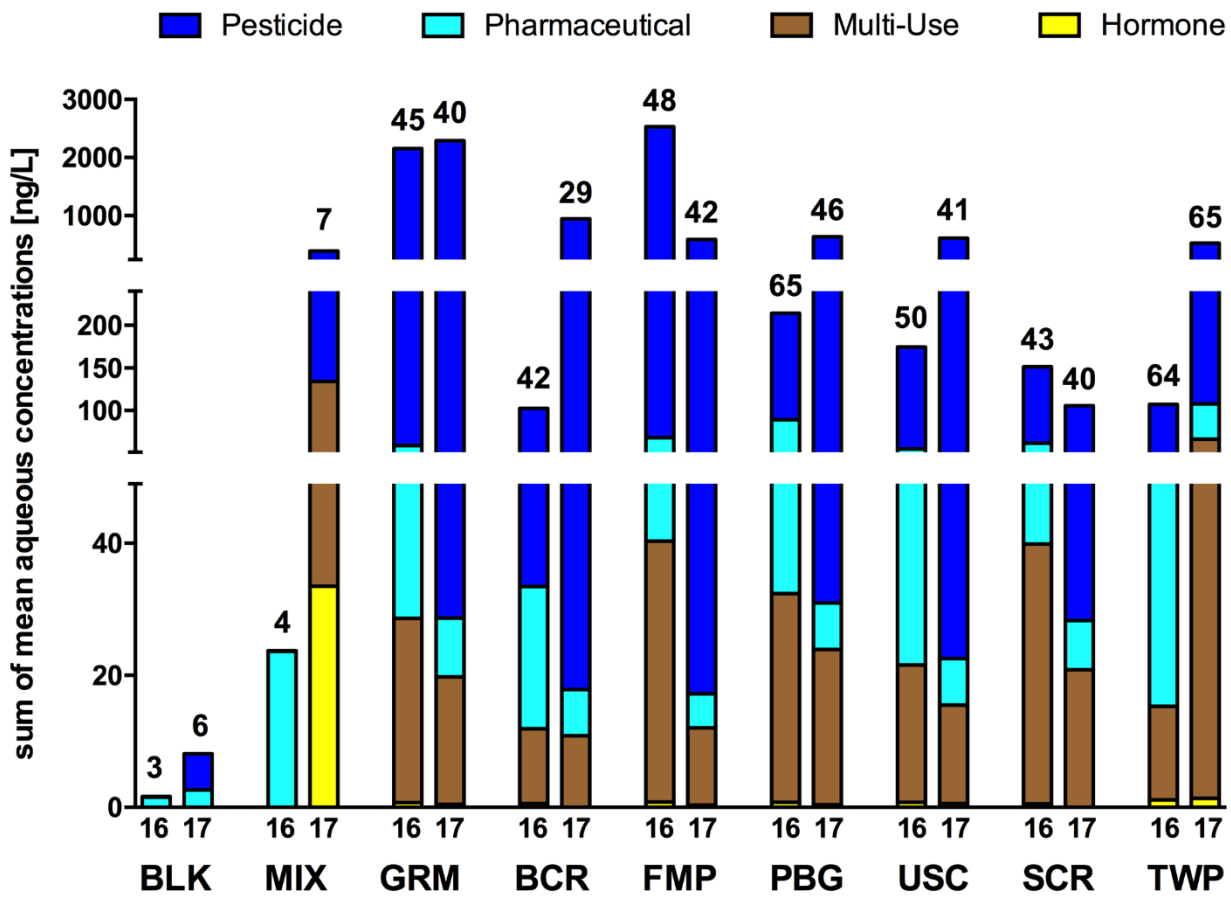

Figure 2.3. Maumee River surface water chemistry for 2016 (left) and 2017 (right) collected during 21-day adult fathead minnow exposures. Treatments listed as negative control (BLK), positive control (MIX), then upstream (GRM) to downstream (TWP) from left to right. Concentrations reported in ng/L, and total number of detected chemicals shown above each site.

Chemical concentrations in water samples were similar between sample years 2016 and 2017, particularly at GRM, FMP, and SCR. Similarly, the number of detections is similar between sample years, particularly at GRM, FMP, USC, SCR, and TWP. In 2016 water samples, pharmaceuticals make up a larger portion of the total chemical load than in 2017 samples. Contrarily, pesticides make up a larger portion of the total chemical load in 2017 water samples as compared to 2016 samples. Multi-use chemicals

generally made up a slightly larger portion of the total chemical load in 2016 water samples as compared to 2017 samples, except at TWP.

When chemical concentrations in water samples are evaluated from an upstream to downstream perspective, the total chemical load is similar between sites. However, pesticides make up a larger portion of the total chemical load in the upstream sites as compared to the downstream sites, while multi-use chemicals and pharmaceuticals make up a larger portion of the chemical load downstream as compared to upstream sites. The blank control water samples had the fewest chemicals detected and lowest concentrations for both sample years. The positive control sample in 2016 had a lower total chemical concentration than any other sample site, while the 2017 positive control sample was lower than multiple sample sites. The difference in total concentrations in positive control water samples may be due to degradation of the 2016 sample.

A total of 10 chemicals (5%) were detected in at least half of the tissue samples, while 37 chemicals (18%) were detected in at least one tissue sample. Only pesticides and pharmaceuticals were detected in tissue samples. The most commonly detected chemicals (detected in at least half of the samples) were: beta BHC (100%, pesticide), o,p'-DDD (100%, pesticide), o,p'-DDE (100%, pesticide), Azithromycin (100%, pharmaceutical), alpha BHC (83%, pesticide), Atrazine (78%, pesticide), Desethylatrazine (78% pesticide), Endosulphan Sulphate (67%, pesticide), Virginiamycin M1 (67%, pharmaceutical), and Carbadox (56%, pharmaceutical).

For male fish, total chemical concentration was similar between sites. However, site FMP had considerably higher total chemical concentrations as compared to the other sites, and the positive control sample had considerably lower total chemical concentrations as compared to the other sites. Total chemical concentrations for male fish primarily consisted of pesticides.

For female fish, total chemical concentrations were more variable between sites than was observed for male fish. Sites GRM, FMP, PBG, and SCR had considerably higher total chemical concentrations than the blank control, positive control, or sites BCR, USC, or TWP. However, similar to male fish, the site with the lowest total chemical concentration in female fish was the positive control. Total chemical concentrations were similar between female and male fish. For female fish, pharmaceuticals comprised a larger portion of the total chemical concentrations measured, as compared to male fish. In particular, at site SCR, pharmaceuticals account for over half of the total chemical concentration for female fish.

2.3.2 Adult Fathead Minnow 21-Day Exposures

Adult fathead minnow survival over the 21-day exposure period was greater than 80% for all treatments in both exposure years except for the TWP exposed fish in 2017 which demonstrated a significant decrease in survival as compared to BLK ($p = 0.0052$). CF differed significantly between treatments in male fathead minnow exposed in 2016 ($F(8, 160) = 2.2731$, $p = 0.0249$) although the Dunnett's post-test was unable to resolve these differences. In 2017, the CF in male fathead minnow differed significantly

between treatments ($F(8, 150) = 3.5641$, $p = 0.0008$) with increases in SCR exposed males as compared to BLK ($p=0.0145$). Similar to male fish, the female CF also differed significantly between treatments in 2016 ($F(8, 157) = 3.6751$, $p = 0.0006$) with decreased CF for females maintained in FMP ($p=0.0045$) and TWP ($p=0.03$) treatments (Figure 2.4). In contrast, the CF for female fathead minnow in 2017 did not differ between treatments ($F(8, 158) = 1.7836$, $p = 0.0839$). A marginally significant effect was observed for the TWP exposed female as compared to BLK (one way ANOVA with Dunnett's post-test, $p=0.0464$).

Expression of SSC in 2016 differed between treatments (one-way ANOVA ($F(8, 160) = 2.5597$, $p = 0.0119$) with greater SSC expression in male fish exposed to waters from USC when compared to BLK (Dunnett's post-test, $p=0.0026$). Male SSC expression in 2017 did not differ between treatments ($F(8, 149) = 1.084$, $p = 0.3774$).

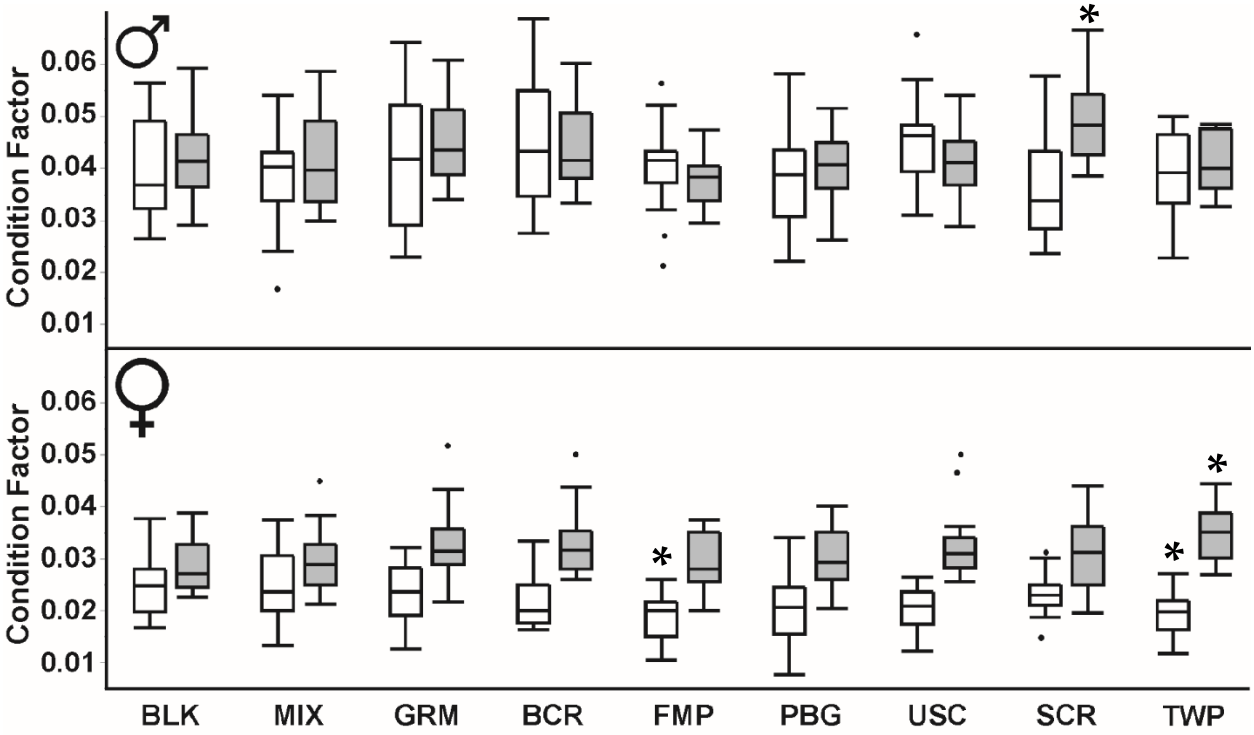

Figure 2.4. Adult fathead minnow CF for 2016 (left, unshaded) and 2017 (right, shaded). Significant differences from negative control (BLK) indicated with *.

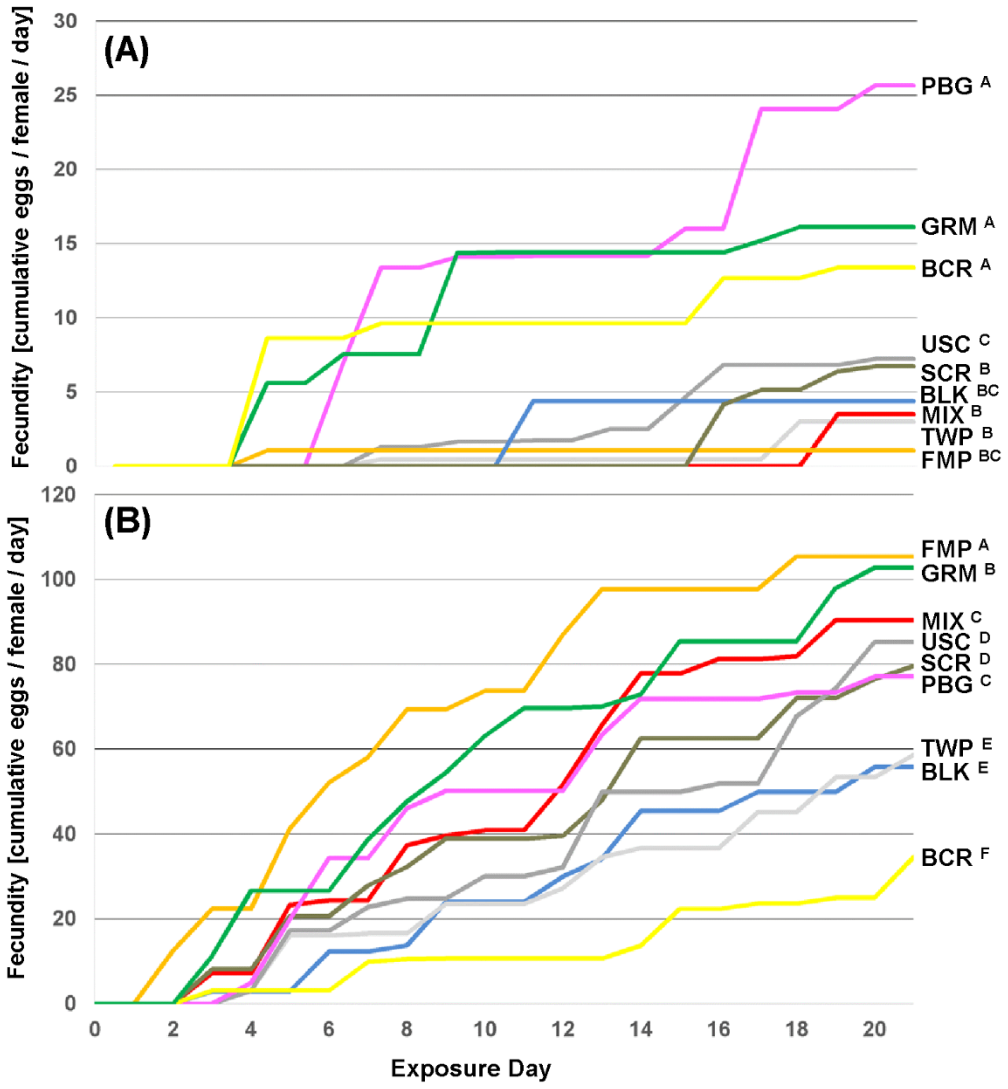

Figure 2.5. Adult 21-day exposure fecundity 2016 (A) and 2017 (B) depicted as the cumulative number of eggs laid per female per day. Significant differences indicated by differing letters following treatment abbreviation.

Fecundity, measured as the cumulative mean number of eggs per female per day, differed significantly between treatments ($F(1.541, 29.279) = 48.011$, $p<0.0001$) and was greater at four of the five sites upstream of the urban area of Toledo in 2016 (GRM, BCR, PBG and USC) when compared to the two downstream sites (Figure 2.5a). The

downstream and control sites (FMP, SCR, TWP, BLK, and MIX) were not statistically different from one another. Fecundity in 2017 also demonstrated significant differences between treatments ($F(1.677, 33.543) = 72.127$, $p<0.0001$) with higher fecundity in fish maintained for 21 days in water from upstream sites (GRM, FMP, PBG). Interestingly, MIX also had higher fecundity than fish in waters from sites further downstream on the Maumee River (USC, SCR, TWP), fish maintained in water from BCR, and fish from the blank control. Among the urban sites, fish in USC and SCR water were more fecund than fish in the TWP, BLK, and BCR treatments (Figure 2.5b).

Male blood glucose did not differ between treatments in fish exposed in 2016 ($F(8, 159) = 1.5304$, $p = 0.1505$). Male blood glucose in 2017 demonstrated significant differences between treatments through a one way ANOVA ($F(8, 145) = 3.6067$, $p = 0.0008$). Yet, the Dunnett's post-test found no significant differences from the BLK control among all treatments. Likewise, female blood glucose in 2017 demonstrated significant differences ($F(8, 152) = 2.0495$, $p = 0.0442$) by a one way ANOVA, but the Dunnett's post-test did not indicate any differences as compared to the BLK treatment.

Male plasma Vtg concentrations differed between treatments in both years. In 2016 ($F(8, 142) = 66.2835$, $p < 0.0001$), plasma Vtg was significantly increased in MIX exposed males ($p<0.0001$) and significantly reduced in FMP exposed males ($p=0.0429$) compared to plasma Vtg concentrations in male fathead minnows in the BLK treatment. In 2017 ($F(8, 142) = 22.7704$, $p < 0.0001$), Vtg was significantly higher only in MIX exposed males ($p<0.0001$).

Male plasma E_2 ($F(8, 157) = 1.672$, $p = 0.1092$), 11-KT ($F(8, 150) = 1.6581$, $p = 0.1132$), and the estrogen:androgen ratio ($F(8, 150) = 1.5576$, $p = 0.1422$) did not differ between treatments in 2016. Similarly, in 2017 male plasma E_2 ($F(8, 140) = 1.3416$, $p = 0.2279$), 11-KT ($F(8, 148) = 1.732$, $p = 0.0955$), and the estrogen:androgen ratio ($F(8, 132) = 1.5954$, $p = 0.132$) did not differ between treatments. A marginally significant effect was observed for the estrogen:androgen ratio for male fish in the 2017 USC treatment (one way ANOVA with Dunnett's post-test, $p=0.0406$).

2.3.3 Larval Fathead Minnow 21-Day Exposures

Survival, as determined by percentage (%) surviving per exposure jar, differed between treatments in 2016 ($F(8, 27) = 4.6221$, $p = 0.0012$) as it was significantly decreased in MIX exposed larvae as compared to BLK ($p=0.0055$). No differences in larval survival were found in 2017 ($F(8, 45) = 1.083$, $p = 0.3922$). The growth of larval fathead minnow differed between treatments in 2016 ($F(8, 306) = 2.2715$, $p = 0.0226$). Unfortunately, the Dunnett's post-test was unable to resolve the differences. 2017 larval growth demonstrated no significant differences between treatments ($F(8, 470) = 1.5025$, $p = 0.1537$).

Latency, the reaction time of a larval fathead minnow to respond to a simulated predator stimulus, was not significantly affected by any treatment exposure in both 2016 ($F(8, 102) = 0.9811$, $p = 0.4552$) and 2017 ($F(8, 144) = 0.2229$, $p = 0.9863$) . Similarly, the escape velocity (normalized to body lengths per millisecond) of larval fathead minnow was not affected by any treatment in 2016 ($F(8, 102) = 0.819$, $p = 0.5876$) or 2017

(F(8, 144) = 1.2034, p = 0.301) . The escape angle of larval fathead minnows was also not affected by any treatment in 2016 (F(8, 102) = 0.7741, p = 0.6264) or 2017 (F(8, 144) = 1.3882, p = 0.2064). However, the Dunnett's post-test indicated an increased escape angle in fathead minnow larvae previously exposed to PBG as compared to BLK exposed larvae (p=0.0238) . The total escape performance, a combination of latency and escape velocity was not significantly altered by any treatment in 2016 (F(8, 102) = 0.3692, p = 0.9346) or 2017 (F(8, 144) = 1.0216, p = 0.4225).

Feeding efficiency of larval fathead minnow, as measured by the percentage (%) of live brine shrimp consumed in a one-minute period, was unaffected in 2016 (F(8, 92) = 0.6039, p = 0.7724) and 2017 (F(8, 148) = 0.3354, p = 0.9511).

2.3.4 Embryonic Fathead Minnow 7-Day Exposures

Viability of embryos did not significantly differ between treatments in 2016. However, 2017 viability of embryos were significantly reduced in MIX (p = 0.0016), FMP (p = 0.0008), and PBG (p = 0.0006) exposed embryos as compared to BLK. The time to hatch for each developing embryo was also analyzed and demonstrated significant differences between treatments in 2016 (F(8, 92) = 31.1691, p < 0.0001). All treatments demonstrated a shorter time to hatch than BLK (p<0.0001). 2017 also demonstrated significant differences between treatments in time for embryos to hatch (F(8, 174) = 12.5688, p < 0.0001) with eggs exposed only to the PBG (p<0.0001) and USC (p<0.0001) treatments demonstrating a shorter time to hatch than BLK. The sum of all larval deformities (1 - normal to 3 - severe) differed significantly between treatments in 2016

($F(8, 92) = 3.0543$, $p = 0.0043$) and 2017 ($F(8, 169) = 5.528$, $p < 0.0001$). Exposed fathead minnow embryos demonstrated a significant increase in the expression of developmental abnormalities in FMP (2016 p=0.0293; 2017 p=0.0004) exposed embryos as compared to BLK.

2.4 Discussion

We conducted replicate fathead minnow exposure experiments with waters from multiple field sites of the Maumee River to assess the contributions of agricultural and urban chemical mixtures on the health and reproductive ability of fish. Analytical chemistry confirmed the presence of a multitude of agricultural and urban chemicals in water samples and in the tissue of fathead minnows. Consequently, fathead minnows exposed to these waters for 21 days exhibited a range of health conditions and fecundity (Table 2.3). At the same time, adult survival as well as embryonic and larval fish exposure were not affected by exposure to site waters.

As expected in an agriculturally dominated watershed, pesticides were commonly detected in water and fish tissue in fish throughout the seven sampling sites, although concentrations of pesticides in water and fish tissues were generally higher in water samples from upstream (agricultural) sites and in fish exposed to these waters. The pesticides detected correspond to those used extensively in row-crop planting of corn and soy, which dominate the landscape in this part of the Upper Midwest of the US. As the Maumee River flows into the Greater Urban Area of Toledo, other chemicals, especially pharmaceuticals, contribute more heavily to the overall contaminant burden

measured in water and fish tissue. Accordingly, fish health and reproductive output differ between sites.

Condition factor, an indicator of overall health and metabolism (Fulton, 1904), often declines as fish are exposed to chemical stressors and demonstrate an overall decline in health (Li et al., 2010). The significant decline of CF in females exposed to FMP and TWP water samples represents potential contaminant sources at each of those sites capable of leading to declines in fish health. Increases in CF, as was found in SCR and TWP exposed fish in 2017 – particularly TWP exposed females, may be a result of the estrogenic compounds within effluent aiding in oocyte maturation. Effluent exposed fish have also demonstrated increased CF (Minarik et al., 2014), potentially due to a greater availability of, and more consistent source of, nutrients.

Table 2.3. Summary of biomarkers altered from negative control (BLK). A plus sign (+) indicating a statistically significant positive change, a minus sign (-) indicating a statistically significant negative change, and a 0 indicating no change in a treatment when compared to BLK. Results for 2016 (left) and 2017(right) are separated by a "/" in each data cell. Female (F) and male (M) only changes denoted where necessary. Change from BLK indicated as + or – denote magnitude of change, not biological benefit.

	BLK	MIX	GRM	BCR	FMP	PBG	USC	SCR	TWP
Adult Fish									
Survival [%]	95/92.5	90/100	87.5/97.5	97.5/86.8	92.5/100	90/97.5	90/97.5	97.5/825	92.5/65
CF		0/0	0/0	0/0	-(F)/0	0/0	0/0	0/+(M)	-(F)/+(F)
SSC		0/0	0/0	0/0	0/0	0/0	+/0	0/0	0/0
Fecundity		0/+	+/+	+/-	0/+	+/+	0/+	0/+	0/0
Glucose		0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Vtg		+/+	0/0	0/0	-/0	0/0	0/0	0/0	0/0
Estradiol		0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
11-KT		0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
E2 / 11-KT ratio		0/0	0/0	0/0	0/0	0/0	-/0	0/0	0/0
Larval Fish									
Survival		-/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Growth		0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Latency		0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Escape velocity		0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Escape angle		0/0	0/0	0/0	0/0	0/+	0/0	0/0	0/0
Total escape		0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Feeding efficiency		0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Embryos									
Embryo viability [%]	79.2/87	100/45.8	91.7/75	100/61.9	100/44.7	66.7/42.6	83.3/79.2	83.3/95.7	83.3/70.8
Time to hatch		-/0	-/0	-/0	-/0	-/-	-/-	-/0	-/0
Sum of deformities		0/0	0/0	0/0	+/+	0/0	0/0	0/0	0/0

Male phenotypic characteristics associated with reproductive dominance, SSC, are known to be controlled by endogenous circulating sex hormones, primarily 11-KT and testosterone (Borg, 1994; Kime, 1998). The expression of more dominant SSC has reproductive benefits, in which males are better able to obtain and maintain a nesting

site as compared to less dominant males (Danylchuk & Tom, 2001). Thus, the increase in SSC expression in males exposed to USC treatment water samples may represent a competitive advantage in which USC exposed males would be better able to reproduce.

The presence of contaminants within a degraded river system have the potential to induce stress related hematological responses (Bevelhimer et al., 2014; Thomas et al., 2017a), including upregulation of blood glucose which serves as an indication of metabolic physiology. The reduction in glucose at two sites in 2017 (PBG and USC) despite greater contaminant load at these sites may represent a therapeutic hazard associated with the chemical footprint at each of these sites. Further hematological analysis of Vtg, an egg-yolk precursor protein expressed in males only in the presence of estrogenic exposure and often used as a bio-marker (Harries et al., 1996, 1997; Purdom et al., 1994), demonstrates the estrogenic nature of the positive control (MIX) used in this experiment as has been previously indicated (Kohno et al., 2017). Despite the known estrogenic potential of many agricultural (Hoskins & Boone, 2017; Xie et al., 2005) and urban/effluent chemicals (Hemming et al., 2004), the lack of upregulation of Vtg in exposed males indicates either concentrations too weak to induce synthesis of Vtg within the Maumee River or potential antagonistic mixture effects countering the estrogenic effects of many known detected estrogenic chemicals. Comparatively, the failure of treatment exposure to significantly alter E_2 and 11-KT expression, as well as their ratio except for one treatment, indicates a system which does not demonstrate significant estrogenic effects during a short-term exposure.

Alterations to adult fathead minnow fecundity, both by year and site location, indicate a system which is heavily influenced by multiple sources and does not demonstrate a dose dependent response of increasing chemical load yielding reduced fecundity. fathead minnow fecundity in 2016 demonstrated higher reproductive output in three of the four most upstream sites (GRM, BCR, and PBG) as compared to all other sites, indicating a contrast between agricultural and urban contaminant loading and its potential impacts on reproduction. However, 2017 demonstrated differences in fecundity that did not depict a clear distinction between agricultural and urban influenced sites, highlighting the importance of seasonal influences on contaminant loading, and differences between lab and field exposures in which increasingly complex mixtures in field settings do not ultimately decrease fecundity as has been demonstrated in laboratory settings (Thrupp et al., 2018). Survival below 80% at only one treatment (2017 TWP) was most likely influenced by the added presence of waste water effluent in combination with other agricultural and urban contaminants. Effluent is known to contain complex chemical estrogenic mixtures (Hemming et al., 2004; Zhang et al., 2017), further adding to the contaminant load of fathead minnow exposed to this treatment.

While adult endpoints are often less sensitive than those associated with developing fathead minnow, the lack of alterations to larval behavioral and physiological endpoints demonstrates little or no threat to developing larvae. Minor alterations to an organisms predator-avoidance behavior can have catastrophic

population level effects (Kidd et al., 2007; McGee et al., 2009; Palace et al., 2009), but the lack of significance to fathead minnow larvae exposed to a degraded and heavily impacted river system demonstrates no perceivable threat to the larval stage.

The occurrence of morphological abnormalities has been well documented in embryos developing in the presence of chemical contamination (Henry et al., 1997; Lefebvre et al., 2004). Thus, the occurrence of increased developmental abnormalities at the FMP site provides an indication of chemical contamination key to embryo development otherwise undocumented at the other six field sites. Similar to embryo development, fertility of eggs can be negatively impacted by the presence of chemical contamination (Nakayama et al., 2004). Decreases in fertilization poses a potential population threat associated with decreased population sizes. Further, decreases in fertilization rates may place additional stressors on adult fathead minnows as they must reproduce at a higher rate to maintain stable populations in the case of decreased fertilization of developing eggs. In addition to morphological and fertilization rates, embryos face an additional threat of predation. The reduction in time to hatch for all treatments in 2016, and for PBG and USC in 2017 represents embryos developing quicker than their negative control counterparts. The significant reduction in time to develop and hatch would likely reduce additional predation threats when developing fathead minnow are at their most vulnerable stages.

Using three fathead minnow life stages, the objective of this study was to determine whether influences associated with heavily degraded agricultural and urban

systems negatively impacted the physiology and reproduction of fathead minnow. All three life stages demonstrated no separation of upstream to downstream effect, nor did a clear separation of agricultural and urban influenced sites occur. Rather, biological effects seemed to be site-specific, with several sites (FMP and USC) demonstrating greater change than both the nearest up- and downstream sites. The occurrence of agricultural and urban contaminants within the Maumee River demonstrates perceivable threats to the fathead minnow, primarily at the adult and embryonic stages, through alterations to reproduction and morphological development. While the fathead minnow remains a lower trophic level species, its known potential as a model species may represent concern for other higher trophic level species.

Chapter 3. Impacts of Agricultural and Urban Land-Use in the Maumee River Watershed on the Anatomy and Physiology of Caged and Resident Sunfish (*Lepomis* Spp.)

3.1 Introduction

The Upper Midwest of the USA is a global leader in row crop and meat production (Hatfield, 2012). This astonishing productivity, 0.8% of the world's agricultural land producing 4.37% of global production ("Agricultural Land," 2018, *World Agricultural Production*, 2018), is driven by high-intensity agricultural practices including the application of fertilizer and pesticides, spreading of manure from concentrated animal feedlot operations, and drain-tiling of farmland. Unfortunately, these agricultural practices may increase chemical contamination of nearby waters and lead to the degradation and impairment of aquatic ecosystems (DeLorenzo et al., 2001; Heaney et al., 2001; Lammert & Allan, 1999). In addition, many watersheds associated with agricultural land uses frequently are impacted by other pollutant sources including those stemming from urban areas which may contribute wastewater effluent, urban runoff, and combined sewer overflows to an already impacted riverine system. These multiple sources of anthropogenic pollutants ultimately result in aquatic ecosystems exposure to complex chemical mixtures (Focazio et al., 2008; Gros et al., 2007; Pal et al., 2010; Wang, 2014). Remediation of observed adverse biological impacts in aquatic ecosystems requires an understanding of the contribution of multiple sources to an aquatic ecosystem. Unfortunately, little is known about the contributions of

multiple pollutant sources to adverse biological outcomes in large riverine systems. In the current study, the Maumee River (Ohio) in the heart of the agricultural farmland of the USA and flowing through the metropolitan area of Toledo, OH was studied to assess whether documented biological effects were a result of cumulative chemical loads or the impact of nearby land use practices.

The Maumee River is the largest US tributary to the Laurentian Great Lakes. It drains an area dominated by high-intensity agricultural practices including row-crops and concentrated animal feedlot operations which pose a threat of pesticide/herbicide pollution as well as livestock specific chemicals (Burkholder et al., 2007; Gilliom, 2007; Richards & Baker, 1993). Just prior to emptying into the western basin of Lake Erie, the river flows through the city of Toledo, Ohio where it receives discharge from storm sewers, surface runoff, as well as industrial and treated municipal wastewater effluent. The presence of urban centers along rivers often has an oversized chemical footprint that is enhanced by runoff during precipitation events, which may result in episodic chemical inputs into aquatic ecosystems (Dwight et al., 2002; Taebi & Droste, 2004; Yong & Chen, 2002; Zhao et al., 2010). The Greater Toledo Area also includes a large industrial complex and harbor which may contribute contaminants to the river through runoff or lack of complete breakdown during wastewater treatment (Suthar et al., 2010). The final source of aquatic pollutants to this systems is wastewater effluent, in which not all chemical compounds are broken down during the treatment cycle (Deblonde et al., 2011; Köck-Schulmeyer et al., 2013; Rosal et al., 2010). Wastewater effluent often

makes up the most consistent contribution of pollution to riverine systems regardless of precipitation, season, or land use (Lee et al., 2011). As a major river to the Great Lakes watershed, and with a history of contamination, the Maumee River is an ideal system in which to study the impacts from multiple land use practices as the land use surrounding the river transitions from agricultural to (sub-)urban, industrial, and finally wastewater effluent inputs.

The complexity of pollutants sources, pollutants mixtures, and multitude of anthropogenic stressors require a two-part experimental approach to examine the contributions of multiple pollutant sources to the documented biological impacts (Dean-Ross & Mills, 1989; Karr et al., 1985; *Maumee Area of Concern Stage 2 Watershed Restoration Plan*, 2006, *Maumee River Basin Area of Concern Remedial Action Plan Recommendations*, n.d.). Using organisms reared under controlled conditions as sentinels to assess the hazard of water sources from different regions of the river representing different land use characteristics allows for the integration of complex pollutant mixtures while excluding non-pollutant anthropogenic confounding variables (such as degraded habitats, invasive species, human activity). While the use of resident organisms provides a more long-term assessment of land use characteristics and the impacts of complex pollutant mixtures. By assessing fish either reared in a controlled laboratory setting and exposed for an acute period or fish exposed for an entire life-cycle, the contribution of each complex pollutant mixture could be analyzed in regards to agricultural, urban, or industrial land use. It was, therefore, expected that agricultural

land would be associated with the presence of pesticides, herbicides (Elliott et al., 2017; Nowell et al., 2018), and livestock specific chemicals (ie. growth promoters, steroids, livestock pharmaceuticals) dependent on the operation (Jaffrézic et al., 2017). Biological effects from agricultural pollutants include feminized sex ratios (Hoskins & Boone, 2017), reductions in fecundity (Jensen et al., 2006), and a reduction in species abundance and total number (Schäfer et al., 2007). In contrast, we expected urban areas to contribute contaminants including heavy metals, nutrients, and suspended solids (Brezonik & Stadelmann, 2002), as well as polycyclic aromatic hyrdocarbons (Van Metre et al., 2000). Potential biological effects include acute toxicity to invertebrate species (Bay et al., 2003), and pre-spawning mortality among mature fish (Scholz et al., 2011). The presence of industrial facilities has been demonstrated to lead to aquatic pollutants including pharmaceuticals (Larsson et al., 2007), surfactants (Field & Reed, 1996), and synthetic sterols (Desbrow et al., 1998). These industrial derived pollutants often lead to biological effects in aquatic organisms such as creating male-biased sex ratios (Larsson & Förlin, 2002), alterations in reproductive physiology (Van den Heuvel & Ellis, 2002), and increases in plasma vitellogenin concentrations indicative of estrogenic consistency (Tremblay & Van Der Kraak, 1999). Lastly, wastewater effluent contain pollutants ranging from pharmaceuticals and personal care products, to insecticides, musks, fragrances, and a variety of other contaminants of emerging concern (Zhang et al., 2017). The biological effects of such a diversity of pollutants may include changes in reproductive output (Thrupp et al., 2018), alterations to embryonic development (Zhang

et al., 2017), and larval survival (Rearick et al., 2014). Adult fish may experience the suppression of male reproductive behaviors (Martinovic et al., 2007) and changes in plasma sex hormone concentrations (Hemming et al., 2001; Jobling et al., 2004).

Seven field sites were selected based on a variety of land uses and potential input characteristics (Figure 2.1; Table 2.1) to assess the biological effects of a degraded river system impacted by agricultural and urban land uses. Sunfish, a native species to most of North America, were used in this study as they represent an important component of the aquatic food chain. Use of the sunfish in two different stages, hatchery reared and short-term exposure *versus* resident and life-long exposure, allowed for the interpretation of potential physiological and anatomical alterations associated with exposure duration. When combined, this multi-faceted data set provides a comprehensive assessment of the contribution to biological impacts of a variety of land-use-dependent pollutant mixtures on fish health.

3.2 Materials & Methods

3.2.1 Study River and Study Sites

The river used for this case study, the Maumee River, represents a historically degraded watershed as a result of agricultural practices and urban development. The Maumee River forms in Fort Wayne, IN, as the confluence of the St. Joseph and St. Mary's rivers before flowing 220km through northwest Ohio and discharging into Lake Erie. The Maumee River watershed is characterized by glacial moraine influenced soils, and prior to settlement was composed of wetlands, forests, and grasslands forming

"The Great Black Swamp" – the last region around the southern Laurentian Great Lakes to be colonized. Converting the Great Black Swamp into productive farmland required extensive drain tiling and the channelization of the Maumee River during the 19th century. Today approximately 80% of the watershed is under agricultural use, primarily dedicated to corn and soybean production while urban land cover accounts for 11% of the Maumee River watershed (*Evaluation of Land Use / Land Cover Characteristics in Ohio Drainages to Lake Erie*, 2008). The altered soil characteristics through agricultural improvements and the draining of the former swamp, the Maumee River has become heavily polluted with sediment and phosphorus (Stow et al., 2015) thus contributes to algal blooms in the western portion of Lake Erie. The extensive agricultural use coupled with an abrupt shift to a large urban population in Toledo, OH, makes the watershed an ideal model to examine the role of an agriculturally degraded and urban influenced river.

Seven study sites were selected to evaluate a gradient of land uses common in the Great Lakes Basin, starting with more agricultural influences upstream and increasing urban influence downstream. Grand Rapids (GRM) was the most upstream site on the main stem of the Maumee River, located immediately above a dam near Grand Rapids, OH. Beaver Creek (BCR) is a small side-stream that drains poultry and dairy farmland before it empties into the Maumee River just downstream of Grand Rapids. The Farnsworth Metro Park site (FMP) was the last site upstream of increasing urban influence, and was located immediately upstream of Waterville, OH on the main

stem of the Maumee River. Perrysburg (PBG) represents the transition from agricultural to sub-urban land use and receives treated wastewater effluent from this small community. All sites downstream of, and including PBG, are located in what will be referred to as the Greater Toledo Area, encompassing urban and suburban Toledo and its surrounding suburbs. A site just upstream of Swan Creek (USC) was chosen to delineate the transition from suburban to urban and industrial land use. Swan Creek (SCR) is a small side-stream that flows mostly through residential urban areas before emptying into the Maumee River in downtown Toledo, OH. Swan Creek also contains multiple combined sewer overflows (CSOs), wastewater treatment plants (WWTPs), and potential storm water runoff from downtown Toledo, OH. The most downstream site, just below the Toledo wastewater treatment plant (TWP), was located less than 2km from the confluence with Lake Erie.

3.2.2 Surface Water Chemical Analysis

Surface water was collected once per week from each site for chemical analysis. Briefly, two liters of water were collected in High Density Polyethylene (HDPE) bottles and three liters were collected in baked amber glass bottles. All bottles were conditioned with site water prior to sample collection. Sampling consisted of collecting surface water grab samples by submerging inverted sampling bottles before filling to avoid any scum present on the water's surface. Samples were cooled and shipped on ice to AXYS Laboratories (Sidney, British Columbia) and Southern Illinois University (Carbondale, Illinois) for chemical analysis.

At AXYS Laboratories, samples were analyzed for a suite of multi-residue pesticides (MREs), hormones, and pharmaceuticals and personal care products (PPCPs). Multi-residue pesticides (AXYS Method MLA-035) were analyzed using high resolution gas chromatography/high resolution mass spectrometry (HRGC/MS) following methods described in EPA Method 1699. Hormones and PPCPs (AXYS Method MLA-075) were analyzed using high performance liquid chromatography reversed phase C18 or HILIC column, coupled to a triple quadrupole mass spectrometer (LC-MS/MS), modified from methods described in EPA Method 1694.

3.2.3 Chemical Data Treatment

Following methods described in Elliott et al. (2017) and Thomas et al. (2017), chemical results were reduced to one sample per site. This reduction was achieved by using the maximum concentration detected for each chemical at every site. Maximum concentrations were used for this study due to the abundance of left-centered data (i.e., non-detects), and due to the limited number of samples per site (n=4/site) that would result in insufficient data for other estimation techniques (e.g., maximum likelihood). Additionally, by using the maximum concentration for a given chemical, that concentration will signify the greatest toxicological concern for fish exposed in this study, which is relevant when evaluating biological endpoints. However, due to the limited sampling effort, it is unlikely that water samples captured the true maximum concentration for all analyzed contaminants. To provide further interpretive ability when evaluating chemical input and comparing to biological endpoints, chemicals were

categorized into four chemical classes based on their commercial use; Hormones, Multi-Use, Pesticides, and Pharmaceuticals. Total chemical concentrations were calculated for each site; first by total chemical concentration for each class for each site, and then total chemical load for each site.

3.2.4 Caged Sunfish Exposure

600 sexually mature, hatchery-reared bluegill sunfish (*Lepomis macrochirus*) were delivered to Toledo, OH in an aerated hatchery truck on the morning of fish deployment (Jones Fish & Lake Management, Cincinnati, OH). A subset of 60 sunfish were dissected upon arrival (see *Biological Analysis* below) for a baseline (BLN) measurement. Sunfish (n=25 sunfish per cage, n=75 per site) were transported to each field site in aerated coolers and placed in three mesh cages (1m length x 0.3m diameter) attached to the river bed (i.e., bottom of cage was in contact with sediment) for a duration of 14 days to assess the effects of short-term exposure. All fish were deployed within four hours of arrival in Toledo. Cages were equipped with a HOBO Data Logger (Onset Computer Corporation, Bourne, MA) to monitor water temperature (°C) every 10 minutes over the duration of the exposure. Caged sunfish were used to analyze individual site-specific effects, as these fish were maintained at one location for a 14-day period, unable to migrate to other habitats.

3.2.5 Resident Sunfish Collection

Concurrently to caging sunfish at each site, resident sunfish were collected using a Smith-Root electro-shocking boat. At each site resident sunfish were collected (n=60)

spanning four species including (1) bluegill sunfish (*Lepomis macrochirus*), (2) green sunfish (*Lepomis cyanellus*), (3) pumpkinseed sunfish (*Lepomis gibbosus*), and (4) orange spotted sunfish (*Lepomis humilis*). Sunfish are a native species to the Maumee River and maintain high site fidelity during the spring spawning season when mature males defend nesting sites (Bartlett et al., 2010; Paukert et al., 2011). Sunfish collection occurred during the reproductive season, which may have helped limit the movement of male sunfish throughout the river system. While bluegill sunfish are common throughout the Maumee River, their abundance varies between sites, making it difficult to obtain 60 per site. Thus, the four most common species were used to achieve a larger sample size. This compromise in experimental design is minimized because of the four species similarities in habitat preferences and phylogenetic closeness (Avise & Saunders, 1984)(Harris et al., 2005), frequent hybridization between species (Avise & Saunders, 1984; Davies et al., 2012), and hybrid offspring which remain fertile (Lagler & Steinmetz, 1957).

3.2.6 Sunfish Biological Processing

Upon collecting both caged and resident sunfish, fish were anesthetized in neutral buffered 0.1% MS-222 (Argent Chemical Laboratories, Redmond, WA). Fish were measured for total and standard length (mm), as well as wet mass (Ohaus Scout Pro 0.1g, Parsippany, NJ). A subset of sunfish (n=20) were saved for chemical analysis (see *Sunfish Chemical Analysis* below). From the remaining sunfish (n=40), plasma was collected by severing the caudal artery and collecting blood using 2 mL microtubes

(Phenix Research Products, Candler, NC). Blood samples were centrifuged at 5,000 rpm for 10 minutes and separated plasma was removed, collected, and stored on dry ice for further analysis. Blood glucose was recorded using a TRUEbalance blood glucose meter (Moore Medical LLC, Farmington, CT) by collecting a blood sample from the severed caudal artery.

Liver and gonad samples were removed from each sunfish and weighed using an Ohaus Scout scale (0.001g, Parsippany, NJ) for calculation of the hepatosomatic (HSI) and gonadosomatic (GSI) indices. Liver and gonad samples were placed in micromesh biopsy processing cassettes (Simport, Beloeil, QC, Canada) and preserved in 10% neutral buffered formalin for later histological analysis.

3.2.7 Sunfish Biological Analysis

In addition to glucose measurements, laboratory analysis of plasma vitellogenin (VtG) was conducted using a competitive antibody-capture ELISA with a sunfish-validated anti-vitellogenin polyclonal antibody and purified sunfish vitellogenin as a standard (Cheek, King, Burse, Borton, & Sullivan, 2004). Analysis followed previously published protocols (Schultz et al., 2013). Biological indices were calculated using the following equations:

CF = [total mass (g) / total length (mm)]

HSI = [liver mass (g) / fish mass (g)] * 100

GSI = [gonad mass (g) / fish mass (g)] * 100

Liver and gonad histological analysis was conducted to determine alterations to liver vacuolization and gonad maturity and followed previously described protocols (Thomas et al., 2017). Briefly, following standard H& E processing (Carson, 1997)(Gabe, 1976), sections were assessed by an experienced histologist (HLS) and ranked on a semi-quantitative scale (1-4) for vacuolization of liver hepatocytes (1 – vacuoles visible in <5% of total area; 2 – vacuoles small but throughout image in <25% of area; 3 – broad presence of large vacuoles in 25% - 50% of area; 4 – vacuoles prominent covering more than 50% of the field of view). The development stage of the gonad (ovary or testis) was also evaluated based on the proportion of cell types visible in the field of view (female: perinuclear oocyte, cortical alveolar, early vitellogenic, late vitellogenic; male: spermatogonia, spermatocyte, spermatid, and spermatozoa). The overall maturity of the sample (on a scale of 1 = immature to 4 = only mature sperm present) was calculated as:

$$\text{Ovary} = ((\%_{\text{primary oocyte}}) + (\%_{\text{cortical alveolar}} \times 2) + (\%_{\text{vitellogenic}} \times 3) + (\%_{\text{mature}} \times 4)) / 100$$

$$\text{Testis} = ((\%_{\text{spermatogonia}}) + (\%_{\text{spermatocytes}} \times 2) + (\%_{\text{spermatids}} \times 3) + (\%_{\text{spermatozoa}} \times 4)) / 100$$

All histological assessments were blinded to eliminate observational bias by the assessor of the tissues. As a quality control measure, we re-assessed a subsample of histological sections a second time and compared the resultant maturity values between the initial and the repeat analysis. The calculated mean maturity values obtained for the two analyses differed by <1%.

3.2.8 Statistical Analysis

Prior to analyses, Vtg data was log (base 10) transformed. Sunfish data was then analyzed using a one-way ANOVA to compare between field sites, followed by a Tukey's post hoc test. Resident and caged sunfish were analyzed separately. Analysis was conducted using SAS JMP Pro Version 13.2.0. A limit of $p < 0.05$ was set for statistical significance.

3.3 Results

3.3.1 Surface Water

Chemicals were detected in every water (refer to Figure 2.3 2016 data only) sample. A total of 37 chemicals were detected in at least half of the water samples, while 110 chemicals were detected in at least one water sample. The most commonly detected chemicals (detected in at least 82% of the samples) were (% of samples with chemical detected, chemical use): Heptachlor Epoxide (94%, pesticide), Atrazine (88%, pesticide), Benzoylecgonine (88%, multi-use), Desethylatrazine (88%, pesticide), Sulfamethoxazole (88%, pharmaceutical), Ametryn (82%, pesticide), Atenolol (82%, pharmaceutical), Caffeine (82%, multi-use), Carbamazepine (82%, pharmaceutical), Cotinine (82%, multi-use), Dieldrin (82%, pesticide), Fluoxetine (82%, pharmaceutical), Metformin (82%, pharmaceutical), Metribuzin (82%, pesticide), Naproxen (82%, pharmaceutical), Triamterene (82%, pharmaceutical), and Venlafaxine (82%, pharmaceutical).

When chemical concentrations in water samples are evaluated from an upstream to downstream perspective, the total chemical load is similar between sites. However,

pesticides make up a larger portion of the total chemical load in the upstream sites as compared to the downstream sites, while multi-use chemicals and pharmaceuticals make up a larger portion of the chemical load downstream as compared to upstream sites.

3.3.2 Biology

Blood glucose in male caged sunfish, as indicated by a one way ANOVA, demonstrated significant differences between sites for caged sunfish ($F(7, 152) = 24.1351$, $p < 0.0001$). A Tukey post-hoc test found that USC exposed sunfish had a statistically significant increase in blood glucose concentrations as compared to all other sites except TWP ($p \leq 0.05$). Sunfish exposed to sites GRM, SCR, and TWP also reported increases in glucose concentrations statistically higher than BLN, BCR, and PBG exposed sunfish ($p \leq 0.05$). FMP sunfish had significantly higher blood glucose concentrations than BLN and PBG sunfish ($p \leq 0.05$). Female caged sunfish likewise had significant differences between sites ($F(7, 157) = 15.1616$, $p < 0.0001$) with sunfish exposed to field site USC having higher blood glucose concentrations than sunfish exposed at all other sites ($p \leq 0.05$). Sunfish exposed at SCR and TWP had significantly higher blood glucose concentrations than BLN, BCR, and PBG sunfish ($p < 0.05$) but were different than sunfish from GRM and FMP. GRM and FMP sunfish had blood glucose concentrations greater than BLN, BCR, and PBG sunfish ($p \leq 0.05$). BLN, BCR, and PBG sunfish were not significantly different. Analysis of male resident sunfish blood glucose between sites resulted in significant differences ($F(6, 186) = 14.462$, $p <$

0.0001). Resident PBG, SCR, and TWP sunfish had statistically higher glucose concentrations than sunfish from GRM, BCR, and FMP ($p \leq 0.05$) but were not different than those from USC. USC sunfish had significantly higher blood glucose concentrations than sunfish from GRM and FMP ($p \leq 0.05$) but were not different than sunfish from BCR. Sunfish from BCR, GRM, and FMP were not statistically different. Blood glucose concentrations in female resident sunfish were significantly different ($F(6, 106) = 6.65$, $p < 0.0001$). Sunfish from TWP had significantly higher blood glucose concentrations than fish from BCR, FMP, and PBG ($p \leq 0.05$) but were not different than GRM, USC, and SCR sunfish. Sunfish from USC and SCR had significantly higher glucose concentrations than sunfish from BCR, FMP, and PBG ($p \leq 0.05$) but were not different than sunfish from GRM. Sunfish from GRM, BCR, FMP, and PBG were not significantly different in blood glucose concentrations. (Figure 3.1, Table 3.1; $p > 0.05$).

Plasma Vtg in male caged sunfish demonstrated no significant differences between treatments ($F(7, 150) = 1.5557$, $p = 0.1529$) while plasma Vtg concentrations in resident male sunfish were significantly differences among sites ($F(6, 173) = 2.1839$, $p = 0.0467$). Sunfish from USC had statistically higher Vtg plasma concentrations than sunfish from SCR ($p = 0.0192$) but were not statistically different from any other sites. All other sites (GRM, BCR, FMP, PBG, and TWP) contain Vtg concentrations which were not statistically different.

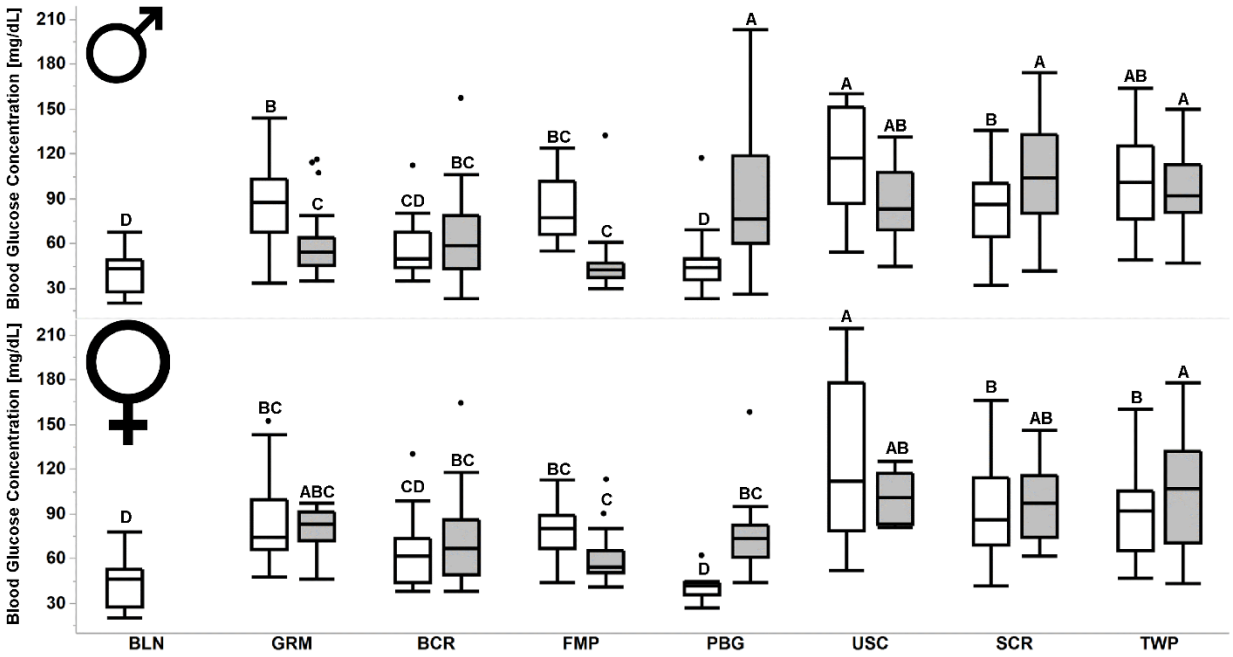

Figure 3.1. Sunfish blood glucose concentrations [mg/dL] with caged (hatchery reared) sunfish – unshaded, and resident sunfish – shaded. Significant differences between treatments indicated by differing letters. Analysis of caged and resident sunfish was conducted separately.

Table 3.1. Sunfish results summary of biomarkers. Depicted using connecting letters report from Tukey's post-hoc test. Male/female response. Green text indicates a beneficial biological response, red text indicated a detrimental biological response.

Caged Sunfish	BLN	GRM	BCR	FMP	PBG	USC	SCR	TWP
Glucose	D/D	B/BC	CD/CD	BC/BC	D/D	A/A	B/B	AB/B
Vtg	A	A	A	A	A	A	A	A
HSI	AB/A	C/C	C/C	BC/C	A/A	BC/C	C/BC	ABC/AB
GSI	A/A	A/C	A/BC	A/BC	A/BC	A/BC	A/BC	A/AB
CF	A/A	B/B	B/B	B/B	B/B	B/B	B/B	B/B
Liver Vacuolization	A/C	A/ABC	A/BC	A/BC	A/A	A/AB	A/ABC	A/AB
Gonad Maturity	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A

Resident Sunfish		GRM	BCR	FMP	PBG	USC	SCR	TWP
Glucose		C/ABC	BC/BC	C/C	A/BC	AB/AB	A/AB	A/A
Vtg		AB	AB	AB	AB	A	B	AB
HSI		AB/AB	B/B	A/AB	AB/A	A/AB	AB/AB	A/B
GSI		A/A	B/AB	AB/AB	AB/A	AB/AB	B/AB	AB/B
CF		B/ABC	C/C	BC/C	B/AB	C/BC	A/A	BC/BC
Liver Vacuolization		A/A	B/A	A/A	B/A	A/A	B/A	B/A
Gonad Maturity		A/A	AB/AB	AB/A	A/AB	A/AB	A/AB	B/B

Significant differences existed in the HSI index in caged male sunfish between sites ($F(7, 163) = 9.8584$, $p < 0.0001$). Male sunfish exposed to PBG had significantly higher HSI indices than sunfish exposed to GRM, BCR, FMP, USC, and SCR ($p \leq 0.05$) but were not significantly different than sunfish exposed to BLN and TWP. Sunfish exposed to BLN had significantly higher HSI indices than sunfish exposed to GRM, BCR, and SCR ($p \leq 0.05$) but were not different from sunfish exposed to FMP, PBG, USC, and TWP. Sunfish exposed at all other field sites (GRM, BCR, FMP, USC, SCR, and TWP) had HSI indices not statistically different. The HSI index in caged female

sunfish also demonstrated significant differences between sites ($F(7, 175) = 12.6711$, $p < 0.0001$). Significantly higher HSI indices occurred in sunfish exposed to BLN and PBG as compared to GRM, BCR, FMP, USC, and SCR ($p \leq 0.05$) but not statistically different from sunfish exposed to TWP. Sunfish exposed to TWP demonstrate significantly higher HSI indices than sunfish exposed to GRM, BCR, FMP, and USC ($p \leq 0.05$) but not statistically different than sunfish exposed to BLN, PBG, and SCR. Sunfish exposed to GRM, BCR, FMP, USC, and SCR demonstrated no statistical difference in their HSI index (Figure 3.2, Table 3.1).

Resident male sunfish were significantly different between sites when analyzing the HSI index ($F(6, 188) = 4.4302$, $p = 0.0003$). Sunfish from FMP, USC, and TWP had higher HSI indices than sunfish from BCR ($p \leq 0.05$) but were not statistically different than sunfish from GRM, PBG, and SCR. The HSI index in resident female sunfish demonstrated significant differences between sites ($F(6, 106) = 4.3082$, $p = 0.0006$). Significant increases in HSI indices occurred in sunfish from PBG as compared to BCR and TWP ($p \leq 0.05$) but no statistical differences between sunfish from PBG and GRM, FMP, USC, and SCR (Figure 3.2, Table 3.1).

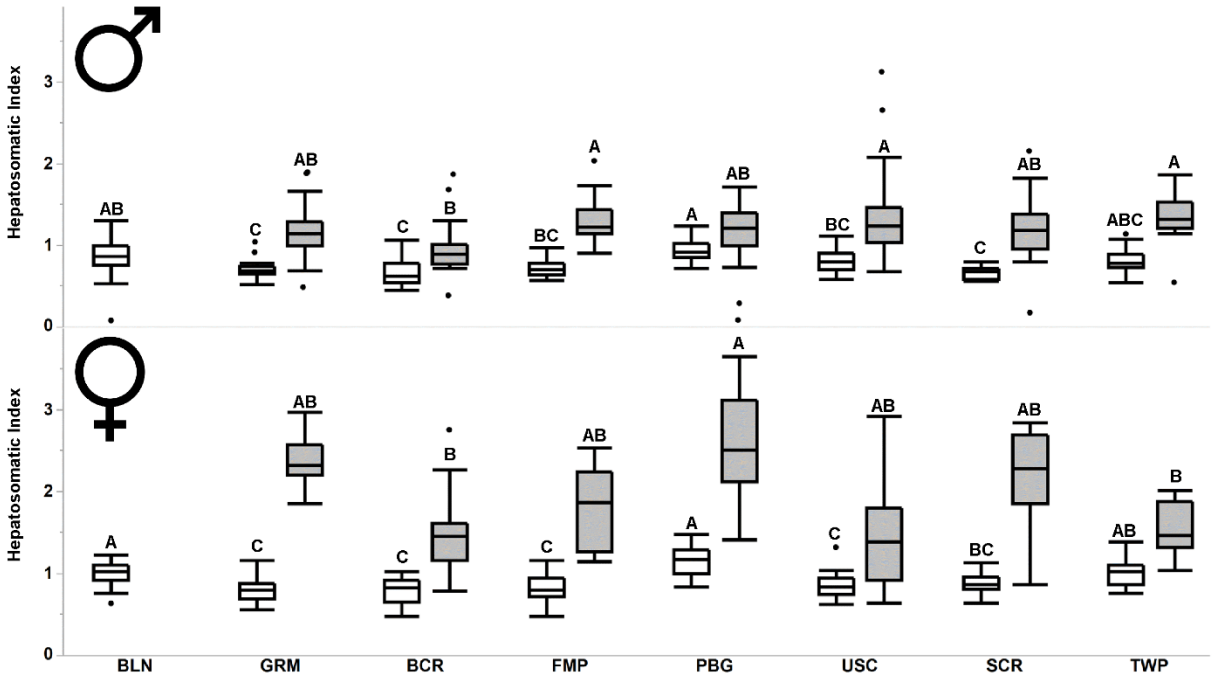

Figure 3.2. Sunfish HSI index depicted as caged (hatchery reared) sunfish – unshaded, and resident sunfish – shaded. Significant differences between treatments indicated by differing letters. Analysis of caged and resident sunfish was conducted separately.

The GSI index in male caged sunfish found no significant differences between sites ($F(7, 155) = 1.3374$, $p = 0.2362$) while significant differences in the GSI index of female caged sunfish existed between sites ($F(7, 161) = 5.7998$, $p < 0.0001$). Sunfish exposed to BLN had statistically higher GSI indices than sunfish exposed to GRM, BCR, FMP, PBG, USC, and SCR ($p \leq 0.05$) but were not statistically different from sunfish exposed to TWP. Sunfish exposed to TWP had significantly higher GSI indices than sunfish exposed only to GRM ($p \leq 0.05$) and were not statistically different from all other treatments. Sunfish exposed to GRM, BCR, FMP, PBG, USC, and SCR were not statistically different. The GSI index in male resident sunfish demonstrated significant differences between sites ($F(6, 186) = 3.8987$, $p = 0.0011$) in which sunfish from GRM

had significantly higher GSI indices than sunfish from BCR and SCR ($p \leq 0.05$) but no statistical differences between sunfish from FMP, PBG, USC, and TWP. The GSI index in female resident sunfish demonstrated significant differences between sites ($F(6, 106) = 3.1815$, $p = 0.0065$). Sunfish from GRM and PBG had significantly higher GSI indices than sunfish from TWP ($p \leq 0.05$) but no statistical differences from BCR, FMP, USC, and SCR.

Caged sunfish exposures for male ($F(7, 229) = 4.1403$, $p = 0.0003$) and female ($F(7, 250) = 7.4392$, $p < 0.0001$) resulted in significant differences between sites for the CF endpoint, with both groups of sunfish exposed to BLN having significantly higher CF's than all other field sites ($p \leq 0.05$). The CF for male resident sunfish demonstrated significant differences between sites ($F(6, 247) = 12.4592$, $p < 0.0001$). Sunfish from SCR has significantly higher CF's than sunfish from all other sites ($p \leq 0.05$). Sunfish from GRM and PBG had significantly higher CF's than sunfish from BCR and USC ($p \leq 0.05$) but were not statistically different than sunfish from FMP and TWP. Sunfish from BCR, FMP, USC, and TWP were not significantly different. The CF for female resident sunfish were significantly different between sites ($F(6, 152) = 8.6838$, $p < 0.0001$). Sunfish from SCR had significantly higher CF's than sunfish from BCR, FMP, USC, and TWP ($p \leq 0.05$) but were not statistically different than sunfish from GRM and PBG. Sunfish from PBG had significantly higher CF's than sunfish from BCR and FMP ($p \leq 0.05$) but were not statistically different than sunfish from GRM, USC, and TWP. Fish from GRM, BCR, FMP, USC, and TWP were not statistically different.

Male caged sunfish exposures resulted in significant differences between sites for liver vacuolization as demonstrated by a one way ANOVA ($F(7, 153) = 2.1206$, $p = 0.0446$) but a Tukey post hoc test found no statistical differences between sites. However, sunfish exposed to PBG were nearing statistical significance for having greater liver vacuolization than BLN exposed sunfish ($p = 0.0521$). Liver vacuolization for female caged sunfish also had significant differences among sites ANOVA ($F(7, 160) = 6.0982$, $p < 0.0001$). Sunfish exposed to PBG had statistically higher presence of liver vacuolization than sunfish exposed to BLN, BCR, and FMP ($p \leq 0.05$) but were not statistically different than sunfish exposed to GRM, USC, SCR, and TWP. Sunfish exposed to USC and TWP had statistically higher liver vacuolization than sunfish exposed to BLN ($p \leq 0.05$) but were not statistically different than sunfish exposed to GRM, BCR, FMP, PBG, and SCR. Sunfish exposed to all other field sites, BLN, GRM, BCR, FMP, and SCR, were not statistically different.

Liver vacuolization for male resident sunfish was found to contain significant differences among sites ($F(6, 185) = 13.8967$, $p < 0.0001$). Sunfish from GRM, FMP, and USC had statistically higher presence of liver vacuolization as compared to all other sites (BCR, PBG, SCR, and TWP; $p \leq 0.05$). Liver vacuolization for female resident sunfish did not differ significantly among sites ($F(6, 103) = 2.1615$, $p = 0.0527$).

Gonad maturity for male ($F(7, 136) = 1.1914$, $p = 0.3118$) and female ($F(7, 159) = 1.4916$, $p = 0.1738$) caged sunfish did not differ significantly among sites. However, gonad maturity for male resident sunfish demonstrated significant differences between

sites (F(6, 180) = 4.1021, p = 0.0007). Sunfish from GRM, PBG, USC, and SCR had statistically higher gonad maturity than sunfish from TWP ($p \leq 0.05$) but were not statistically different than sunfish from BCR and FMP. Gonad maturity for female resident sunfish, likewise, had significant differences between sites (F(6, 104) = 3.0787, p = 0.0082). Sunfish from GRM and FMP had statistically higher gonad maturity than sunfish from TWP ($p \leq 0.05$) but were not statistically different than fish from BCR, PBG, USC, and SCR (Figure 3.3 and 3.4, Table 3.1).

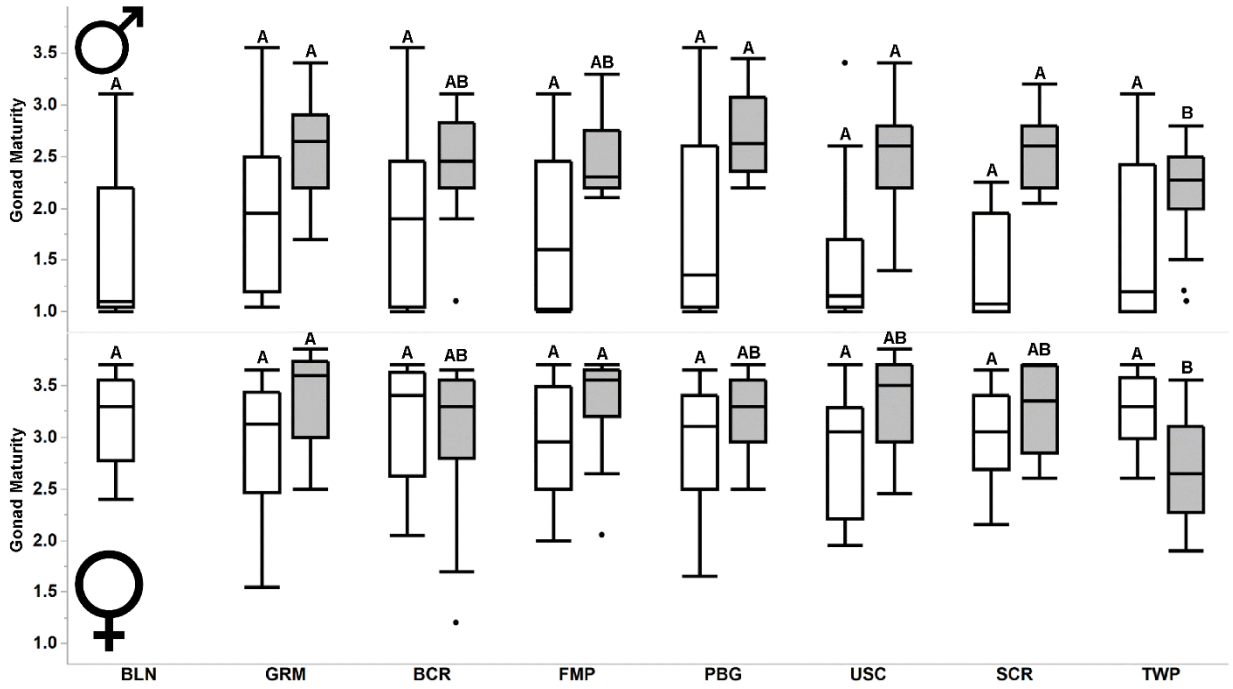

Figure 3.3. Sunfish gonad maturity depicted as caged (hatchery reared) sunfish – unshaded, and resident sunfish – shaded. Significant differences between treatments indicated by differing letters. Analysis of caged and resident sunfish was conducted separately.

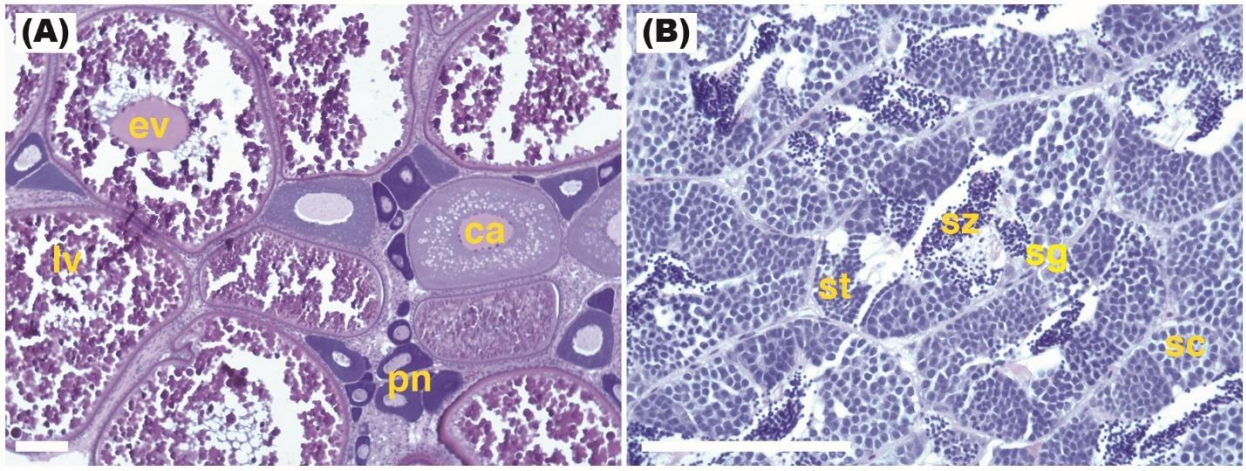

Figure 3.4. Histological section of female (A) and male (B) gonad. Ovary developmental stages indicated as **pn** (primary oocyte), **ca** (cortical alveolar), **ev** (early vitellogenic), and **lv** (late vitellogenic mature). Testis developmental stages indicated as **sg** (spermatogonia), **sc** (spermatocytes), **st** (spermatids), and **sz** (spermatozoa).

3.4 Discussion

Chemical analysis confirmed the presence of a multitude of agricultural and urban chemicals in water samples. As expected in an agriculturally dominated watershed, pesticides were commonly detected in water throughout the seven sampling sites, although concentrations of pesticides in water were generally higher in water samples from upstream (agricultural) sites. The pesticides detected correspond to those used extensively in row-crop planting of corn and soy, which dominate the landscape in this part of the Upper Midwest of the US. As the Maumee River flows into the Greater Urban Area of Toledo, other chemicals, especially pharmaceuticals, contribute more heavily to the overall contaminant burden measured in water. Accordingly, fish health and biological endpoints differ between sites.

The presence of contaminants within a degraded river system have the potential to trigger stress induced hematological responses (Bevelhimer et al., 2014; Carvalho & Fernandes, 2008; Gül et al., 2004; Thomas et al., 2017a), including upregulation of blood glucose which serves as an indication of metabolic physiology. Significant upregulation of blood glucose in both caged and resident sunfish at more downstream sites highlights the potential for urban and industrial based pollutants, as well as residual agricultural pollutants from upstream sources, to cause a greater metabolic stress response in sunfish. As compared to upstream exposed sunfish, in which pollutant sources were more heavily influenced by agricultural practices alone, downstream exposed fish (USC, SCR, and TWP) within the higher intensity urban land-use most likely demonstrated an increased glucose response to a more varied source of chemicals from urban runoff, waste water effluent, combined sewer overflows, and the sum of upstream agricultural contaminants.

Minor alterations to Vtg synthesis, a bioindicator of estrogenic contaminant exposure (Harries et al., 1996, 1997; Purdom et al., 1994), within USC resident male sunfish demonstrate potential differences to total estrogenic loads as compared to other sites. Estrogenic contaminant loads often increase in the presence of waste water effluent (Hemming et al., 2004; Zhang et al., 2017), and although not toxic to male fish, the presence of Vtg indicates alterations to energy allocation at an otherwise energy-intensive reproductive period. Overall higher concentrations of plasma Vtg in caged versus resident sunfish for all treatments may be due to the energetic cost of Vtg

synthesis. In order for sunfish to synthesize Vtg, energy must be allocated away from other somatic processes as has been explained by Aruke & Goksøyr (2003). It is thus hypothesized that hatchery-reared sunfish maintain higher energy reserves necessary for the overall higher synthesis of plasma Vtg.

Opposite the effect of Vtg, the overall increase in the HSI index of both male and female resident sunfish as compared to caged sunfish is expected from native fish which have undergone a complete life-cycle of exposure to aquatic pollutants, among other aquatic stressors. Blood glucose, a metabolic stress indicator, increased in downstream sites while the HSI index did not follow the same effect. Rather, as is consistent between both caged and resident, as well as male and female sunfish, there existed individual site effects indicative of acute stressors present at sites such as PBG. In the presence of acute chemical exposure, liver mass increases in response to the need for additional glycogen used in chemical detoxification of an organism, thus leading to increases in the HSI index (Fanta et al., 2003; Stehr et al., 1998). The expression of increased HSI in both caged and resident sunfish at identical sites reinforces the use of hatchery reared organisms to better understand environmental conditions at any given location.

The GSI , used as an indicator of sexual maturity, often decreases in fish in the presence of contaminant exposure (Hassanin et al., 2002; Lei et al., 2013; McMaster et al., 1991), and thus it serves as a good indicator of chemical contamination and potential reproductive output of organisms at any given site. Reduction in caged female sunfish

GSI, as compared to baseline (BLN) sunfish, indicates organisms which are less sexually mature after only two weeks of exposure. The occurrence of significant reductions after only two weeks highlights the severity of pollutants within the Maumee River capable of altering anatomy in an acute time frame. Within resident sunfish, both male and female, the significant increase in GSI from GRM sunfish as compared to several downstream sites indicates that the additional contaminant load of downstream exposed organisms has a significant effect on sexual maturity. While there are both known and unknown routes of pollution at more downstream sites, the decrease in sexual maturity in resident sunfish is consistent with pollutant related changes in caged sunfish and suggests threats to the sexual development of exposed organisms.

The CF, a good overall indication of fish health (Fulton, 1904), demonstrated matching patterns in male and female caged sunfish. An overall decrease in CF from all field exposed sites, as compared to BLN sunfish, demonstrates the overall stress associated with contaminants sunfish are exposed to, regardless of site. Resident sunfish likewise demonstrated differences among CF, with both male and female sunfish from SCR demonstrating higher CF than other sites. SCR is a tributary to the main channel of the Maumee River. Increases in CF in fish from SCR indicates the elevated level of stress placed upon fish within the more heavily impacted main channel of the Maumee River. The increased presence of liver vacuolization in caged sunfish, as compared to BLN sunfish, further demonstrates the extent to which both high intensity agricultural and urban land use practices impact biota in the Maumee

River. Increases in liver vacuolization associated with contaminant load (Roy & Bhattacharya, 2006; Teh et al., 1997) demonstrates that individual sites across the lower stretch of the Maumee River are capable of improperly allocating resources, potentially impacting the resultant CF for any given sunfish. Further, resident male sunfish demonstrated alterations to liver vacuolization inconsistent with the effect of elevated glucose at more downstream sites, highlighting the level of impact both agricultural and urban land use play in the health of aquatic organisms. While endpoints such as glucose indicate elevated stress on organisms in more downstream/urban settings, most endpoints (i.e., HSI, GSI, CF, and liver vacuolization) demonstrate the complexity of inputs into the Maumee River and further support the need for targeted, site-specific exposures to better understand the contaminant input at any given location.

Employing both caged (hatchery reared) and resident sunfish in the field analysis of the Maumee River improved the interpretation of the acute and chronic impacts of contaminant pollution. This effect is highlighted in the differences between caged and resident sunfish in their biological responses in which resident sunfish appear to demonstrate both more, and more severe alterations, to biological changes. In the case of gonad maturity, gonad maturity was not altered in caged sunfish while resident sunfish experienced a decline in sexual maturity in fish exposed at the most downstream site (TWP). This alteration at a site heavily impacted by wastewater effluent is consistent with other studies demonstrating greater alterations in fish exposed to effluent (Bjerregaard et al., 2006; Jobling et al., 2002; Orrego et al., 2006).

Effluent exposed fish have been shown to delay the progression of spermatogenesis in male fish (Jobling et al., 2002), result in testes development presenting only the early developmental stage, spermatogonia, (Bjerregaard et al., 2006), and lead to increases in gonadal maturity in female fish as compared to reference sites (Orrego et al., 2006). The implications of reduced sexual maturity in resident sunfish may include a longer time to sexual maturity (Gao et al., 2009) and thus a lower reproductive output over the course of an organisms life, as well as a potential threat to the population level of resident organisms (Kidd et al., 2007; Palace et al., 2009).

Using both caged and resident sunfish, an analysis of the Maumee River and its associated land use characteristics highlighted the severity of human influence on aquatic health. Endpoints within both acute and chronic exposed organisms depict a system heavily influenced by immediate upstream inputs capable of inducing stress and physiological related responses in exposed organisms. The use of caged and resident sunfish also highlighted the differences in exposure length, with resident sunfish demonstrating more severe biological responses more indicative of true environmental parameters. It can thus be concluded that the degraded land use within the Maumee River watershed adversely impact the anatomy and physiology of the sunfish, a key aquatic species within Great Lakes tributaries.

Chapter 4. Agricultural Contaminant Mixtures at Measured Environmental Concentrations Alter Reproduction in the Second Exposure Generation

4.1 Introduction

There are $1.65*10^6$ km^2 of high intensity farm land in the United States, of which a quarter is found in the Upper Midwest and Great Lakes region ("Major Land Uses," 2018). High intensity agriculture relies heavily on the application of fertilizer and agricultural chemicals which has resulted in increasing aquatic pollution (Moss, 2008; Novotny, 1999; Schwarzenbach et al., 2010). Although many studies have chronicled the adverse biological effects of single chemicals in laboratory settings (Hoskins & Boone, 2017; Jensen et al., 2006; McGee et al., 2009) and the population level effects in field studies (Kidd et al., 2007; Palace et al., 2009; Schäfer et al., 2007), little is known about the long-term consequences of agricultural contaminant mixtures on the health of fish populations. The current study investigated the reproductive effects of agricultural contaminant mixtures across multiple generations in a controlled fathead minnow (*Pimephales promelaes*) laboratory study.

The use of agricultural chemicals in high intensity crop production is necessary to attain sufficient yields in which crops are grown in high density monoculture settings (Tilman, 1999). However, the use of agricultural chemicals may lead to the unintended pollution of surrounding aquatic environments with mixtures of agricultural chemicals (Heaney et al., 2001; Nowell et al., 2018) through runoff, erosion, and groundwater contamination (Holt, 2000; Rozemeijer et al., 2010). Mixtures of contaminants of

emerging concern (CEC) in the aquatic environment often contain herbicides (Gilliom, 2007) and livestock-specific chemicals (ie. growth promoters, livestock pharmaceuticals) (Burkholder et al., 2007; Jaffrézic et al., 2017). All agricultural CECs pose a threat to aquatic environments, regardless of their intentional use, through either runoff or sediment erosion (Holt, 2000). In addition to crop and livestock specific CECs, other non-agricultural chemicals are often detected in waterways downstream of agricultural lands (Elliott et al., 2017). Other commonly detected CECs may include estrone, a mammalian bi-product excreted in the urine of mammals, bisphenol-A (BPA), an estrogenically active plasticizer used frequently in plastic products, N,N-diethyl-m-toluamide (DEET), the most common insect repellant, Tris(2-butoxyethyl) phosphate (TBEP), an inactive ingredient in most herbicide applications which functions as a flame retardant, and alkyl phenols, commonly used in herbicide applications as surfactants (Elliott et al., 2017; Kolpin et al., 2002; Thurman et al., 1992). The present study used data from a Laurentian Great Lakes wide water sampling effort that encompassed 22 of the largest tributaries (Brigham et al., 2015). The focus was thus to determine the multi-generational biological effects of environmentally measured concentrations of agricultural CECs in a commonly used laboratory fish species, the fathead minnow (*Pimephales promelas*).

Field studies focused on the biological effects of agricultural CECs have demonstrated reductions in species abundance and total numbers in impacted environments (Schäfer et al., 2007). While field studies demonstrate true ecosystem-

wide alterations, they fail to identify the effects solely associated with agricultural CEC exposure. In laboratory settings, the exposure of aquatic organisms to single chemical agricultural CECs has resulted in decreased survival and elevated cortisol stress responses (Waring & Moore, 2004), increases of the egg-yolk precursor protein in male fish (Bringolf et al., 2004), as well as feminized sex ratios (Hoskins & Boone, 2017). In addition to physiological alterations, agricultural CECs demonstrate behavioral changes within exposed organisms, including changes in habitat preference (Steinberg et al., 1995), alterations to larval swimming patterns and activity levels (Alvarez & Fuiman, 2005), as well as effects to larval predator avoidance behaviors (McGee et al., 2009). While physiological and behavioral responses alone may not pose an ecosystem level threat, reductions in reproductive output may threaten population levels. The exposure of aquatic organisms to agricultural CECs has demonstrated reductions in fecundity in the presence of feedlot contaminants (Jensen et al., 2006), estrogenic bi-products (Thrupp et al., 2018), and various herbicides (Papoulias et al., 2014; Villarroel et al., 2003). While the occurrence of single chemical laboratory exposures has demonstrated adverse biological responses, short-term single chemical exposures fail to address two key issues: (1) the effects of agricultural chemical mixtures and (2) the effects of multi-generation exposure.

While short-term single chemical exposures present a standard method of assessing biological consequences of exposure, they lack ecological relevance as they underestimate the complexity of chemical mixtures and as they expose organisms only

during a single (often adult) life stage. The present study addressed both issues through a multi-generational approach using mixtures of environmentally measured agricultural chemicals. Using a multi-generational approach better allows for environmental relevance as organisms are exposed throughout their entire life-cycle, capturing sensitive stages of development otherwise avoided in short-term adult exposures. Further, multi-generational studies allow for analysis across generations within the same study, providing insight into the effects of exposure window and duration of exposure (first generation – exposed only during adult stage *versus* second generation – total life exposure). Use of fathead minnows provides a model which reproduces quickly and frequently, and also logistically provides the benefit of completing a multi-generational exposure within a shorter period of time as compared to other fish species. Fathead minnows have been extensively studied and are well understood (Ankley & Villeneuve, 2006), making them an ideal model for a multi-generational exposure. CECs can alter initial molecular and/or physiological responses, causing adverse responses leading to behavioral changes, often triggering population level changes through reductions in reproduction (Jensen et al., 2006; Papoulias et al., 2014; Thrupp et al., 2018; Villarroel et al., 2003) and altered population dynamics (Kidd et al., 2007; Palace et al., 2009). Through a multi-generational study, endpoints focused within all three areas (physiology, behavior, and reproduction) can be examined and allow for better interpretation of ecosystem level threats.

The objective of the current study was to determine the biological consequences of environmentally measured agricultural CEC mixtures within the context of a multi-generational exposure utilizing fathead minnows as the model species. This approach will allow for greater environmental relevance due to the use of agricultural chemical mixtures as well as the complete life-cycle exposure of our model organism. It is hypothesized that F exposed to environmentally relevant agricultural CEC mixtures will demonstrate (1) alterations to biological responses detrimental to both the organism (physiology and behavior) and population level (reproduction) and (2) more severe biological responses in generations (2/3) exposed during their entire life-cycle as compared to generations (1) exposed only during their adult life-cycle.

4.2 Materials and Methods

A three-generation exposure was conducted using fathead minnows over the course of ~11 months. The first exposed generation, F1, consisted of mature adult fathead minnows (>5 months old) exposed during only their adult life-stage for 60 days. Offspring were collected to propagate F2 which were reared until 6 months of age (sexually mature) at which time they were paired for a reproductive period. Their offspring, F3, were reared only through sexual differentiation, for a total of 3 exposed generations (Figure 4.1).

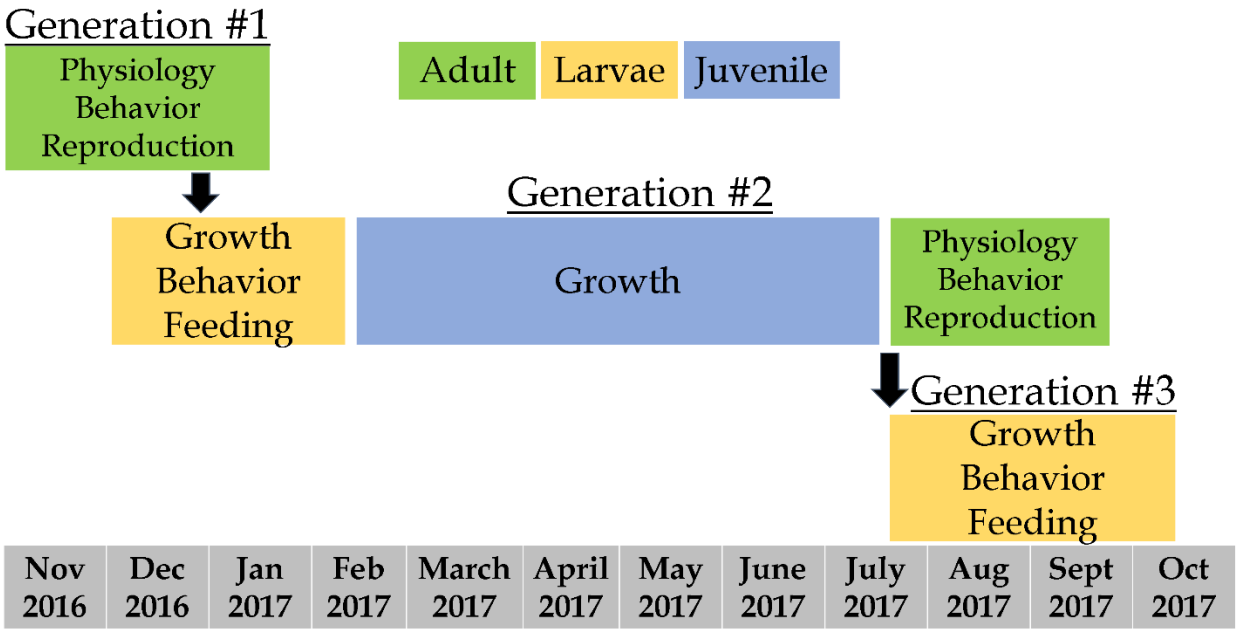

Figure 4.1. Laboratory multi-generational exposure timeline with analyzed endpoints (duration: 321 days).

4.2.1 Chemistry

The creation of the agricultural mixture was completed through an analysis of field validated water chemistry. Water sampling during the years 2013-14 was conducted at 24 of the Laurentian Great Lakes' tributaries (Figure 4.2). Grab samples of surface water were collected spanning stretches of river associated with both agricultural and urban land use as well as spring and summer seasons. Water chemical analysis was conducted at the USGS National Water Quality Laboratory (Denver, CO) as previously described (Thomas et al., 2017a) to identify common co-occurring contaminants. ArcGIS was utilized to assess land use at each of the field collection sites characterizing each site as either agricultural or urban land use. All contaminants detected in ≥30% of grab samples were included in the laboratory mixture, with the

highest detected environmental concentration used as the medium concentration. A ten-fold increase from medium concentrations was used as a high treatment, while a ten-fold dilution of the medium concentration was used as a low treatment. Eight co-occurring agricultural chemicals were used in the laboratory exposure, including: (1) atrazine, (2) metolachlor, (3) bromacil, (4) estrone, (5) bisphenol-A (BPA), (6) N, N-diethyl-meta-toluamide (DEET), (7), tributoxyethyl phosphate (TBEP), and (8) alkyl phenols. USGS National Water Quality Laboratory created mixtures, with stock solutions dissolved in 100% ethanol (EtOH). A solvent control (using 100% EtOH) and a true blank (blank) control were also employed.

Water samples (collected in duplicate) were collected every 9 days throughout the exposure in amber vials (20 mL Amber Borosilicate Vial, C&G Containers, Inc., Lafayette, LA) and frozen immediately. Confirmatory chemistry was conducted at the USGS National Water Quality Laboratory to obtain exposure concentrations.

Exposure aquaria were monitored twice weekly using a YSI (Pro 1020, YSI Incorporated, Yellow Springs, OH) for dissolved oxygen (mg/L and %), temperature (°C), and pH. Exposure were 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.

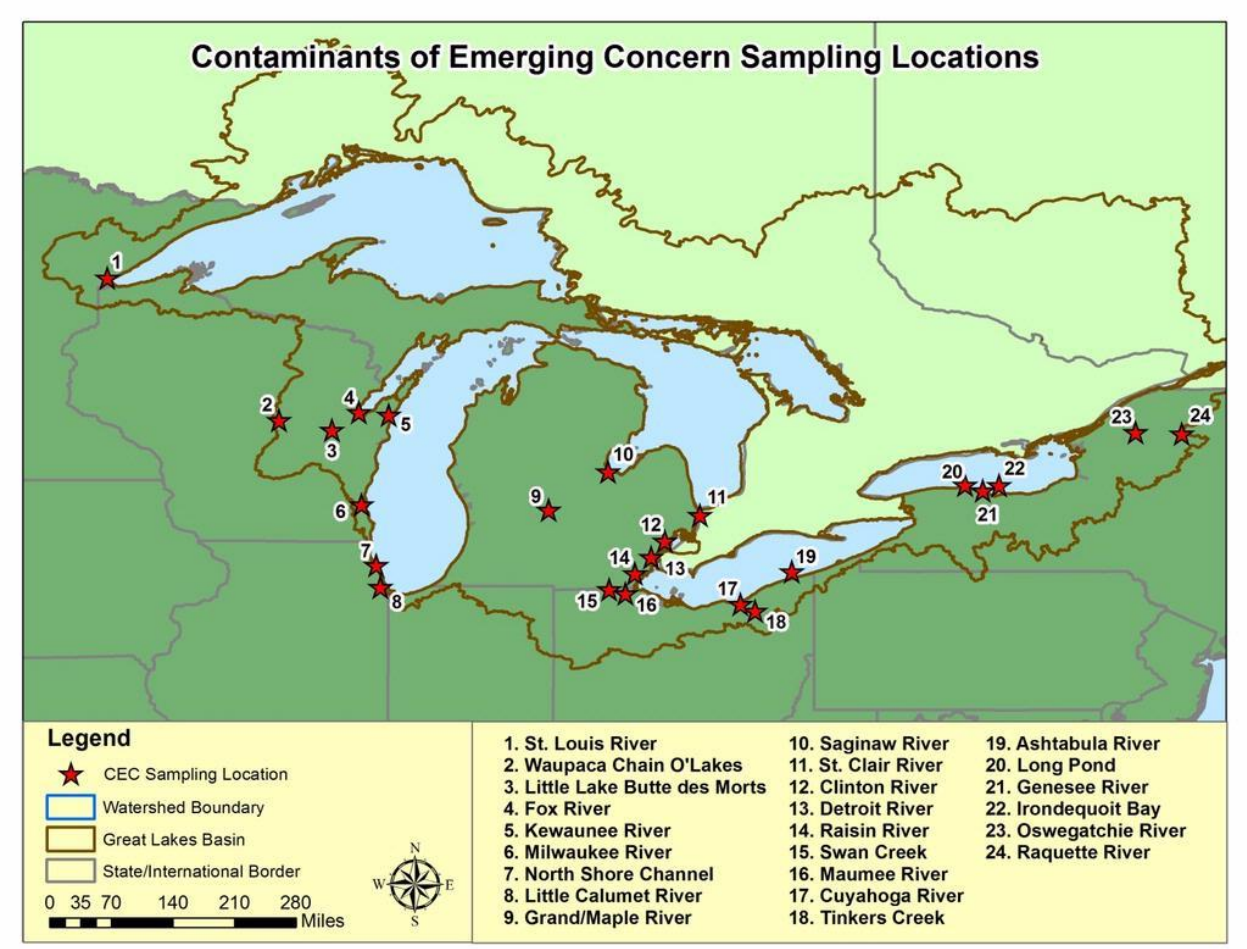

Figure 4.2. Sampling locations of 24 Great Lakes tributaries analyzed in developing environmentally measured concentrations for laboratory multi-generational exposure.

4.2.2 Exposure Design

Fish were housed in a flow-through laboratory facility at St. Cloud State University (IACUC approval permit # 8-82; 8-107) in which three successive generations of fathead minnows were continuously exposed over ~11 months. The flow through system allowed for continuous chemical exposure to minimize chemical losses due to chemical degradation which might occur in a static renewal system. A pre-mixed solution of chemical stock dissolved in 100% EtOH (3 mL) was dissolved in 10 L of DI

water in an opaque carboy (3 Gallon Carboy Glass, Northern Brewer, St. Paul, MN), one carboy per mixing tank. This solution was then pumped to a stainless-steel mixing tank above exposure aquaria via a peristaltic pump (MasterFlex L/S 7519-06, Cole-Parmer, Vernon Hills, IL) at a rate of 2.5 mL/minute. In addition to the chemical stock, heated and filtered well water (22-24 °C) was pumped into the mixing tanks at a rate of 200 mL/minute controlled using flow gauges (Valved Acrylic Flow Meter, Cole-Palmer, Vernon Hills, IL) to allow chemical stock to mix before being dispensed via gravity to exposure aquaria. Exposure water then dripped 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 one of five aquaria (Tygon S3, Pentair Aquatic Ecosystems, Apopka, FL) under each head tank at a rate of 40 mL/minute. Exposure aquaria housed two sets of spawning pairs with a stainless-steel perforated grid separating the two pairs. Aquaria held 20 L of water, allowing for a turnover rate of approximately 3 total water exchanges per 24 hours. Treatments were run in duplicate (2 mixing tanks per treatment) for a total of 20 spawning pairs per treatment (n=20 males, n=20 females per treatment). EtOH solvent control (0.000343 %v/v) was run in triplicate with four exposure aquaria per mixing tank for a total of 24 spawning pairs (n=24) and the blank well water control contained 14 exposure aquaria for a total of 28 spawning pairs (n=28).

F1 sexually mature (5-6 months old) cultured adult fathead minnows were obtained from Environmental Consulting & Testing, Inc. (Superior, WI) in November

2016 (Figure 4.1). Fathead minnows were randomly paired with one male and one female per aquaria. Each aquarium contained a spawning tile made of dense core PVC for reproduction and was aerated (Sweetwater Air Diffusers AS1, Pentair Aquatic Ecosystems, Apopka, FL) to maintain acceptable dissolved oxygen levels (>5 ppm). Aquaria were placed over a metric grid for use in behavioral testing analysis. F1 adults were maintained for a 60 day period consisting of feeding, twice daily *ad libitum*, using a 2:1 mixture of adult brine shrimp:blood worms (Brine Shrimp Direct, Ogden, UT) and daily checks for survival. After 20 days of exposure, fathead minnow spawning tiles were checked daily for fecundity and fertility (days 21-60) in which fathead minnow embryos were hatched and reared in mesh baskets hung in exposure aquaria. Larval fathead minnows were likewise fed, *ad libitum*, twice daily using live hatched brine shrimp (Brine Shrimp Direct, Ogden, UT). After 60 days exposure, adult fathead minnows were euthanized and dissected while their offspring (F2 larvae) grew until July 2017 when they were all 6-7 months of age and sexually mature. F2 fathead minnows were paired, avoiding pairing of related fish, for another 40-day fecundity and fertility period (exposure days 251-290). On exposure day 291, all adult F2 fathead minnows were euthanized and dissected and their offspring were maintained through October 2017 (exposure day 321) to ensure sexual differentiation had been achieved before a subset of each clutch were preserved for sex ratios.

4.2.3 Biological Endpoints

Adult fathead minnows were euthanized with 0.1% MS-222 (Argent Chemical Laboratories; Redmond, WA) prior to dissections (F1 – exposure day 60, F2 – exposure day 291). Fathead minnows were measured for length and wet mass (Ohaus Scout Pro 0.1g, Parsippany, NJ) for use in calculating condition factor (CF) which was calculated using: 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 for each of the tubercles, dorsal pad, and banding coloration as modified from Parrott et al. (2003). SSC were analyzed as the sum of the three characteristics on a scale from 0-9. The tail of all adult fathead minnows was then severed and a TRUEbalance blood glucose meter (Moore Medical LLC, Farmington, CT) was used to obtain a blood glucose reading (mg/dL). Any reading below the detection limit of the reader (values below 20 mg/dL) were transformed as 50% of the lowest detection limit (10mg/dL). Blood was collected from the caudal artery using heparinized micro-hematocrit capillary tubes (Fisher Brand, Pittsburgh, PA) and centrifuged at 5,000 rpm for 5 minutes, after which the percent hematocrit was recorded, and plasma was collected and frozen for later vitellogenin (Vtg) analysis. Laboratory analysis of plasma Vtg was conducted using a competitive antibody-capture ELISA following Parks et al. (1999) for plasma vitellogenin (Vtg) quantification.

Standard preparation and sample analysis followed previously described methods (Minarik et al., 2014).

The liver and gonad of each adult fathead minnow was removed and weighed (Ohaus Scout 0.001g, Parsippany, NJ) for calculation of indices: (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 future histological analysis.

Adult fathead minnows were analyzed during the reproduction periods (F1 – exposure day 49, F2 – exposure day 265) for behaviors necessary for successful defense of mating territory. Adult behavioral trials were recorded using a GoPro 5 (GoPro, Inc., San Mateo, CA) and scored after the completion of the experiment. Nest defense included having both resident male and female fathead minnows in the aquarium and an intruding male was placed in the aquarium in a glass jar. GoPro video recordings were assessed for a suite of male fathead minnow behaviors necessary for successful defense of a nesting site under environmental conditions (Supplemental SOP 4.1).

During reproductive periods of both F1 (exposure days 21-60) and F2 (exposure days 251-290) generations, spawning tiles were checked daily to record fecundity (total number of eggs laid per female per treatment). Spawning tiles with eggs were returned

to aquarium and checked 3 days later to assess fertilization through eye spot counting. Tanks with reproduction were marked to avoid disturbing the fathead minnows until tiles were removed after three days. After three days, and fertilization check, tiles were placed in suspended mesh baskets hung in exposure aquarium to allow eggs to hatch and to prevent predation from adult fathead minnows.

On day 21 post hatch for each clutch of larval fathead minnows, larvae were monitored for growth, exposed to a predator avoidance behavioral trial, and underwent a feeding assay to test foraging behavior. The predator avoidance behavior, 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 larvae 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. Larvae were placed in wells (1 larvae 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 251). During this period, growth was assessed monthly by taking photographs of 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, 4, 5, and 6. During the F2 juvenile growth period, juveniles were culled from aquarium at exposure day 144 to achieve a maximum of 6 juveniles per aquarium in each treatment. Culled larvae were placed in micromesh biopsy processing cassettes and preserved in 10% neutral buffered formalin for later histological analysis to determine sex ratios.

4.2.4 Statistical Analysis

Biological endpoints (except fecundity) were analyzed using a Jonckheere-Terpstra rank-based trend test considering the ordered increase in exposure concentration (IBM SPSS Version 22.0). Only the solvent (EtOH) control was used in the Jonckheere-Terpstra analysis. The true negative control (blank) was compared to the solvent control using a t-test. Unless noted otherwise in the results section, no significant differences existed between the negative and solvent control. Vtg data was first log transformed before statistical analysis was conducted. Fecundity data was analyzed using a repeated measures ANOVA with a Greenhouse-Geisser correction. Statistical significance level was set at $p \leq 0.05$.

4.3 Results

4.3.1 Water Chemistry

Table 4.1. Agricultural multi-generational nominal exposure concentrations [ng/L] including eight chemicals used in mixture and chemical use. Medium concentration is representative of environmentally measured concentration.

Chemical	Chemical Use	Low Concentration [ng/L]	Medium Concentration [ng/L]	High Concentration [ng/L]
Metolachlor	Herbicide	17	170	1,700
Atrazine	Herbicide	40	400	4,000
Bromacil	Herbicide	12	120	1,200
DEET	Insect Repellant	20	200	2,000
Estrone	Mammalian Estrogen	2.4	24	240
BPA	Plasticizer	6	60	600
TBEP	Flame Retardant	210	2,100	21,000
Alkyl Phenols	Surfactant	18.8	188	1,880

4.3.2 Adult Fathead Minnows

A Jonckheere-Terpstra test for ordered alternatives showed that there was no significant trend of increasing or decreasing CF with higher concentration exposure for F1 males (T_{JT} = 1,199, z = 0.06, p = 0.952) or F1 females (T_{JT} = 612.5, z = -1.792, p = 0.073). Similarly, there was no significant trend for F2 males (T_{JT} = 1,554, z = -0.889, p = 0.374) and F2 females (T_{JT} = 1,415, z = 0.03, p = 0.976) regarding the effect of increasing concentration exposure on CF.

The sum of SSC in adult male minnows, analyzed on a subjective scale from 0-9, showed no significant trend in F1 males at increasing concentration (T_{JT} = 1,227.5, z = 0.312, p = 0.755). However, F2 males exposed their entire life-cycle did have a

statistically significant trend of decreasing SSC expression at increasing concentration exposure (T_{JT} = 975, z = -4.658, p < 0.0001) (Figure 4.3).

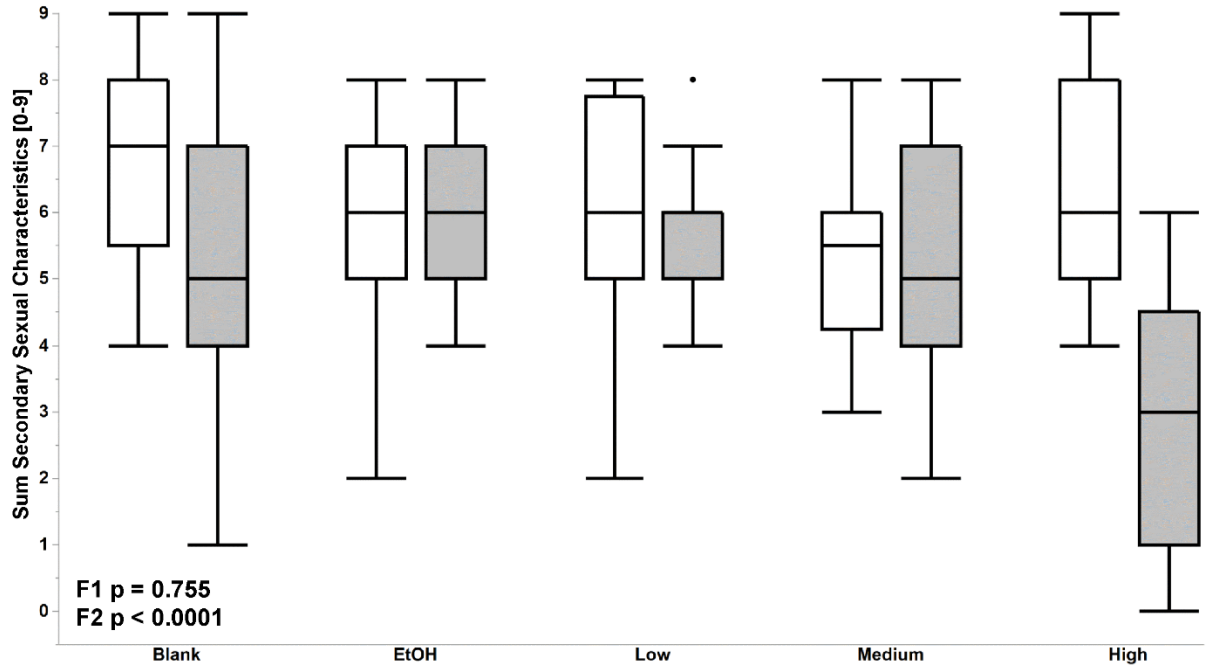

Figure 4.3. Sum of secondary sexual characteristics [0-9]. F1 males depicted on left (unshaded) and F2 males on right (shaded). Statistically significant trend indicated by p-value associated with generation (F1 or F2).

Blood glucose, an indicator of metabolic stress, had no significant trend associated with increasing concentration for F1 males (T_{JT} = 1,118.5, z = 0.391, p = 0.696). For F1 females, blood glucose concentrations in EtOH exposed fathead minnows were significantly higher than those in blank exposed fathead minnows (p = 0.0051); however, there remained no significant trend (T_{JT} = 633, z = -1.312, p = 0.189). Likewise, F2 males (T_{JT} = 1,853, z = 1.612, p = 0.107) demonstrated no significant trend regarding increasing concentration, but again F2 EtOH exposed females had significantly higher

blood glucose concentrations than blank exposed fathead minnows (p = 0.0002). Again, there was no trend of increasing blood glucose concentrations at increasing chemical concentration (T_{JT} = 1,338.5, z = -0.551, p = 0.582).

Hematocrit, the percentage of fish's blood composed of red blood cells, did not result in any significant trend at increasing concentrations in F1 males (T_{JT} = 1,022, z = -1.466, p = 0.143) and F1 females (T_{JT} = 657, z = -1.016, p = 0.309). In F2 males, increasing exposure concentrations did not result in a significant trend of reduced hematocrit (T_{JT} = 1,602, z = -0.57, p = 0.569) but F2 blank exposed males did exhibit significant differences as compared to EtOH males (p = 0.0198). F2 female hematocrit demonstrated no significant trend associated with increasing concentration (T_{JT} = 1,557.5, z = 1.115, p = 0.265).

An analysis of plasma Vtg resulted in a significant trend of increasing plasma Vtg at increasing concentration in F1 males (T_{JT} = 1,772, z = 4.997, p < 0.0001; High exposed males mean Vtg = 484.67µg/mL, EtOH exposed males mean Vtg = 9.48µg/mL) but no significant trend in F1 females (T_{JT} = 773.5, z = 0.744, p = 0.457) at increasing concentration. Like F1 exposed males, the F2 exposed males exhibited a statistically significant trend of increasing plasma Vtg concentrations at increasing chemical concentration (T_{JT} = 2,668, z = 6.871, p < 0.0001; High exposed males mean Vtg = 422.99µg/mL, EtOH exposed males mean Vtg = 7.09µg/mL), while F2 females did not demonstrate a significant trend of increasing plasma Vtg at increasing chemical concentrations (T_{JT} = 970, z = 1.8, p = 0.072) (Figure 4.4).

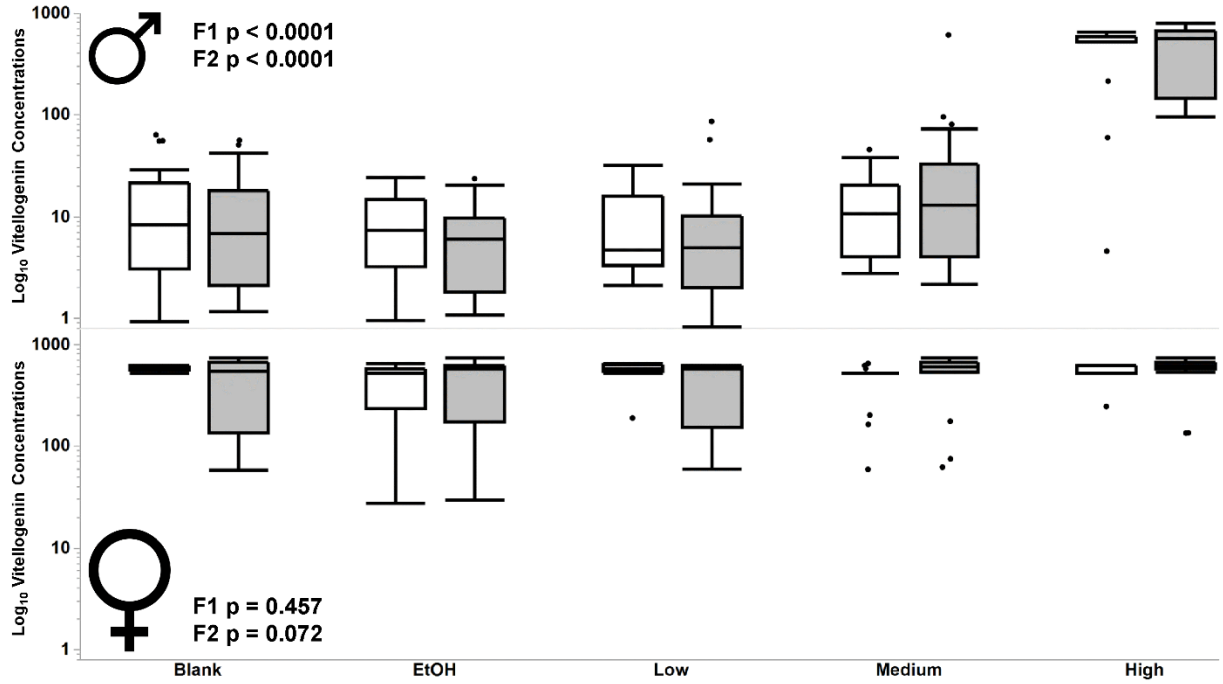

Figure 4.4. Vitellogenin concentrations (depicted as Log_{10}) for male and female adult fathead minnows. F1 adults on left (unshaded) and F2 adults on right (shaded). Statistically significant trend indicated by p-value associated with generation (F1 or F2).

The HSI of both F1 males (T_{JT} = 1,361, z = 1.456, p = 0.145) and F1 females (T_{JT} = 712, z = -0.595, p = 0.552) did not depict a significant trend at increasing concentrations. However, F2 males did have a significant trend of increasing HSI as concentration increased (T_{JT} = 2,022, z = 2.227, p = 0.026). F2 females had no significant trend at increasing concentration (T_{JT} = 1,260.5, z = -1.143, p = 0.253). Analysis of the second anatomical index, GSI, resulted in significant differences in F1 males between EtOH and blank exposed fathead minnows (p = 0.0417). There existed no significant trend at increasing concentrations (T_{JT} = 1,216.5, z = 0.211, p = 0.833). In addition, F1 females (T_{JT} = 717.5, z = -0.529, p = 0.597), F2 males (T_{JT} = 1,881, z = 1.288, p = 0.198), and F2 females

(T_{JT} = 1,548, z = 1.041, p = 0.298) also had no significant trends associated with increasing concentration exposure.

Nest defense, a reproductive behavior expressed in male fathead minnows necessary for successful defense of a mating site, was analyzed for seven variables in both F1 and F2 adult males including: (1) latency to first entrance by the resident male, (2) the total number of approaches to the intruding male, (3) the total time spent within 3cm of the intruding male, (4) the number of lateral displays demonstrating banding coloration, (5) the time to contact the intruding male in the jar, (6) the number of contacts with the intruding male, and (7) the total time interacting with the intruding male. All variables within the nest defense assay were found to lack significant trends associated with increasing concentration exposure over both the first and second generations.

Fecundity in F1, adult only exposed fathead minnows, had significant differences between treatments ($F(2.607, 101.675) = 134.218$, $p < 0.0005$) with medium exposed fathead minnows having the highest overall fecundity, statistically higher than any other treatment ($p < 0.0005$) despite ending in total fecundity below low exposed minnows. Blank and low exposed fathead minnows demonstrated fecundity not statistically different ($p = 1.0$), but both significantly higher than EtOH and high exposed fish ($p < 0.0005$). EtOH exposed minnows had fecundity which was significantly higher than only high exposed fish ($p < 0.0005$) (Figure 4.5A). The life-long exposure of minnows (F2) to the agricultural mixture resulted in significant differences

between treatments ($F(1.224, 48.944) = 88.433$, $p < 0.0005$). Low exposed fathead minnows had the highest overall fecundity, significantly more than all other treatments ($p < 0.0005$). EtOH exposed fathead minnows had the second highest fecundity output and were significantly higher than blank, medium, and high exposed fish ($p < 0.0005$). Medium and high exposed fathead minnows contained fecundity that were not significantly different ($p = 0.114$), but each were significantly higher than blank exposed fathead minnows (medium $p < 0.0005$, high $p = 0.001$). F2 exposed minnows within all treatments containing EtOH (EtOH, low, medium, high) had an overall increase in total fecundity as compared to F1 minnows (Figure 4.5B).

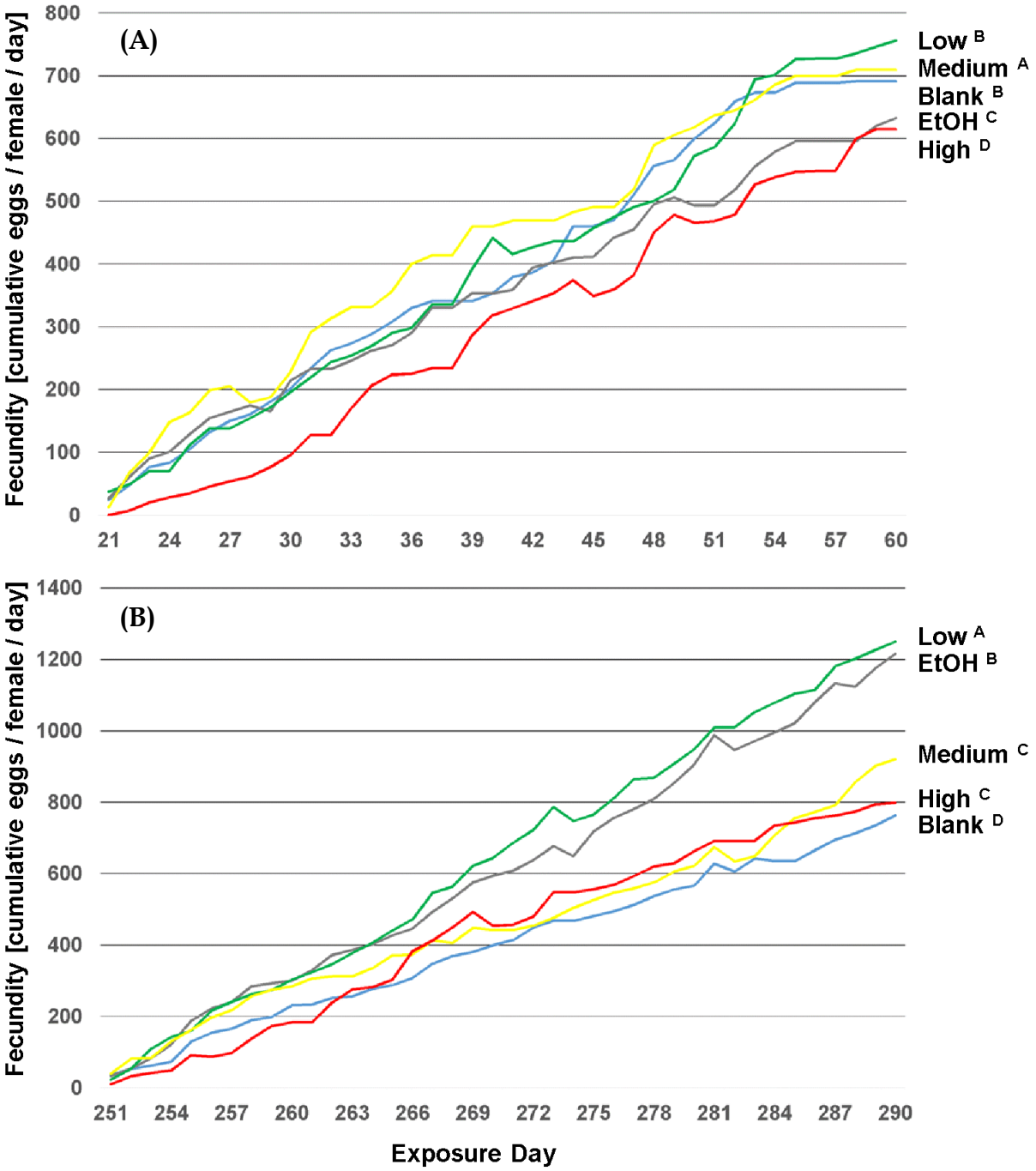

Figure 4.5. F1 (A) and F2 (B) fecundity depicted as the cumulative mean number of eggs per female per day. Significant differences among treatments indicated by differing letters following treatment label.

Table 4.2. Adult biological endpoints demonstrating significant positive (↑) and negative (↓) trends at increasing concentration, or no effect (-). *Fecundity was not analyzed using a trend test, but F2 fecundity did result in significant decreases in at higher concentrations as analyzed by a repeated measures ANOVA.

Adult Endpoints	F1		F2	
	Male	Female	Male	Female
CF	-	-	-	-
SSC	-	N/A	↓	N/A
Glucose	-	-	-	-
Hematocrit	-	-	-	-
Vtg	↑	-	↑	-
HSI	-	-	↑	-
GSI	-	-	-	-
Nest Defense Behavior	-	N/A	-	N/A
Fecundity	N/A	-	N/A	↓*

4.3.3 Larval Fathead Minnows

Larval growth, as measured at 21 days of age during predator-avoidance and feeding efficiency assays, demonstrated no significant trend in F2 larvae (T_{JT} = 9,659, z = -0.213, p = 0.831). F3 blank exposed larvae were significantly longer than EtOH exposed larvae (p = 0.0055), and there was a significant trend in F3 larvae of increasing growth at increasing concentrations (T_{JT} = 7,261, z = 3.442, p = 0.001).

Predator-avoidance behavior consisted of four variables as previously described by McGee et al. (2009): (1) latency, (2) escape velocity, (3) escape angle, and (4) total escape response. The exposure of larval minnows to agricultural mixtures resulted in no significant trends in both F2 and F3 larvae for all four predator-avoidance behaviors.

Larval feeding efficiency was not found to result in significant trends related to increasing concentration exposure in both the F2 (T_{JT} = 7,052.5, z = -1.614, p = 0.106) and F3 larvae (T_{JT} = 5,767.5, z = 0.612, p = 0.541).

4.3.4 Juvenile Fathead Minnows

The monthly growth of F2 juvenile was monitored and resulted in no significant trends at day 21 (T_{JT} = 9,659, z = -0.213, p = 0.831) or months 2 (T_{JT} = 39,231, z = 0.681, p = 0.496), 3 (T_{JT} = 15,075, z = 0.164, p = 0.869), 4 (T_{JT} = 10,418.5, z = -0.134, p = 0.894), 5 (T_{JT} = 9,838, z = -0.616, p = 0.538) or 6 (T_{JT} = 10,786, z = -1.808, p = 0.071) related to increasing concentration exposure. Note that month 5 juvenile blank exposed fathead minnows demonstrated significantly higher growth then EtOH exposed fathead minnows (p = 0.01228).

4.4 Discussion

Using a three-generational fathead minnow exposure to a mixture of commonly co-occurring agricultural chemicals (Elliott et al., 2018), we were able to better understand the environmental consequences associated with long-term exposure. In general, second generation exposed minnows demonstrated more severe biological responses, most likely representing true environmental responses.

Although chemical exposure has been shown to decrease the CF of exposed fish (Li et al., 2010), the lack of response in both generations represents no/minimal alteration to the metabolic health of exposed organisms, as measured by CF (Fulton, 1904). While CF represented no significant trends associated with increasing

concentration, exposed F2 males did demonstrate significant decreases in expression of SSC at increasing concentrations. Male phenotypic characteristics associated with reproductive dominance, SSC, are known to be controlled by endogenous circulating sex hormones, primarily 11-KT and testosterone (Borg, 1994; Kime, 1998). The expression of more dominant SSC has reproductive benefits, in which males are better able to obtain and maintain a nesting site as compared to less dominant males (Danylchuk & Tom, 2001). The lack of alterations in F1 males highlights the differences in generations regarding timing and length of exposure. Previous studies have demonstrated significant SSC alterations in short-term exposures (Martinovic et al., 2007; Miles-Richardson et al., 1999), however the alterations only in F2 exposed males may indicate the chemical mixture used has minimal impact in the short-term on SSC expression, but under continuous environmental exposures, exposed males may be at a disadvantage in maintaining nesting sites.

The use of glucose as an indicator of stress has been previously noted (Bevelhimer et al., 2014; Carvalho & Fernandes, 2008; Gül et al., 2004; Thomas et al., 2017b) in which organisms exposed to increasing contaminant loads upregulate glucose. The lack of upregulation of glucose indicates no increasing stress response across all treatments at increasing chemical concentrations. Alterations to blood glucose concentrations may indicate changes to resource utilization, similar to blood hematocrit. Hematocrit, the percent of blood composed of red blood cells, which can be assessed as a non-specific indicator of health representing an organism's ability to maintain osmotic

homeostasis and respiratory function (Davies, 1987; Lehtinen et al., 1990; Barham et al., 2006; Venkateshwarlu et al., 1990). The lack of a significant trend represents organisms unaffected in their respiratory function as a result of increasing chemical concentrations. While it has been demonstrated that male fish have increased sensitivity in their hematocrit response in the presence of estrogenic compounds as compared to females (Kramer et al., 1998), the concentration and composition of environmentally measured concentrations appears to cause no respiratory stress to either male or female fathead minnows

Further hematological analysis of Vtg, an egg-yolk precursor protein expressed in males only in the presence of estrogenic exposure and often used as a bio-marker (Harries et al., 1996, 1997; Purdom et al., 1994), highlights the potency of agricultural chemicals in which both F1 and F2 exposed males demonstrated significant upregulation. This upregulation, similar to that of glucose, does not pose a toxic threat to male fathead minnows, but may represent improper resource allocation to produce this energetically expensive plasma protein.

The HSI biological index increases in the presence of acute chemical exposure in which liver mass increases in response to the need for additional glycogen used in chemical detoxification of an organism (Fanta et al., 2003; Stehr et al., 1998). The lack of increases in HSI indices in F1 exposed minnows, but present in F2 male minnows, again highlights the differences in biological responses between generation one and two. The HSI index indicates that the agricultural chemical mixture used may not represent an

acute threat, rather, responses associated chemical detoxification are only realized in life-long exposed organisms. The GSI index, used as an indicator of sexual maturity, often decreases in fish in the presence of contaminant exposure (Hassanin et al., 2002; Lei et al., 2013; McMaster et al., 1991). However, the lack of changes within adult fish indicates no perceived threat to the sexual maturity of fathead minnows exposed to environmentally measured concentrations.

The use of behavioral testing within adult, reproductively active fathead minnows, allows for the interpretation of chemical exposure to act regarding known reproductive behaviors. Environmental stimuli, often in the form of chemical contaminants, maintain the potential to act via neural networks to alter physiological responses, ultimately resulting in changes to normal behaviors (Weber & Spieler, 1994). Alterations to normal behaviors have been well documented in the presence of both urban and agricultural contaminants, often leading to changes in survival, the ability to recognize and perceive prey and/or predators, and swimming performance (Scott & Sloman, 2004). The nest defense behavior assessed within this experiment, necessary within male fathead minnows to obtain and maintain a nesting site, has been well documented (Sargent, 1989; Unger, 1983). Despite alterations to SSC within exposed males, a trait beneficial for successful nest defense behavior (Danylchuk & Tom, 2001), no effects occurred in the presence of the agricultural chemical mixture, indicating no perceived harm to a male's ability to maintain a nesting site.

Despite the lack of behavioral alterations within adult exposed minnows, changes which are hypothesized to alter reproductive behaviors and ultimately feedback to environmental consequences (Scott & Sloman, 2004), there existed differences in reproductive output as measured by fecundity. F1 adults exposed to environmentally measured concentrations had the highest overall fecundity as compared to all other treatments, indicating that the overall estrogenic nature of the mixture (Kohno et al., 2017) may appear to play a beneficial role at lower chemical concentrations. Despite increased fecundity at the low concentration, other impacted endpoints (ie. SSC expression, HSI, Vtg synthesis) highlight the potential negative consequences of low level estrogenic exposure including impacted molecular processes and resource allocation. At higher concentrations however, the effects of the estrogenic mixture lead to reduced fecundity. Increasing concentration exposure can alter molecular and physiological changes which can disrupt reproductive processes, resulting in reductions to fecundity (Nash et al., 2004; Villeneuve et al., 2007). While F1 exposed organisms indicate no detriment at environmentally measured concentrations, the effect was not maintained into the second generation. Significant reductions occurred in F2 fathead minnow fecundity in both medium and high treatments, as compared to EtOH and low exposed minnows. This reduction in environmentally measured concentrations within the F2 generation highlights the need for total life-cycle exposure to obtain environmentally realistic outcomes more accurately. Further, the

decrease in environmentally measured concentrations presents a population level threat due to a reduction in reproductive output.

A solvent control effect was also observed in the F2 generation, in which all treatments, including the EtOH solvent control, had a marked increase in fecundity output as compared to the F1 generation, while the blank treatment fecundity output was similar between F1 and F2 generations. Alterations to fecundity with the use of an EtOH solvent have been previously documented (Oliveira-Filho et al., 2009; Zhang & Baer, 2000). The use of EtOH as a solvent also had significant effects, as compared to blank exposed fish, within this study on the endpoints of adult blood glucose, hematocrit, GSI, nest defense behaviors, and larval and juvenile growth. It has previously been hypothesized that the effects of EtOH as a solvent may result in alterations from blank exposed organisms due to one of three pathways: (1) an alteration to gonadal steroidogenesis, (2) alterations to the metabolism of sex steroids which would result in a reduction of 17β-estradiol and thus provide negative feedback on the pituitary, or (3) EtOH acting as an additional nutrient source for micro-organisms and/or bacteria (Hutchinson et al., 2006). However, the concentrations of EtOH used in this study (3.4 µL/L well water) were well below the recommended 20 µL/L put forth by Hutchinson et al. (2006), and thus warrant further study to determine any potential impacts of using EtOH as a solvent control.

While adult endpoints are often less sensitive than those associated with developing fathead minnow larvae, the lack of alterations to larval behavioral and

physiological endpoints demonstrates little or no threat to developing larvae. The presence of increasing growth in F3 larvae was most likely an effect of density-dependent growth and not due to chemical exposure. Minor alterations to an organisms predator-avoidance behavior can have catastrophic population level effects (Kidd et al., 2007; McGee et al., 2009; Palace et al., 2009), but the lack of significance to fathead minnow larvae exposed to a complex agricultural chemical mixture demonstrates no perceivable threat to the larval stage. Similarly, the lack of alterations to juvenile growth indicates no threat to the juvenile stage. Differences in growth at the 5-month age point between blank and EtOH exposed fish may have been a result of density-dependent growth as blank treatments on average contained less juvenile fish (~3.5) per aquarium than the EtOH treatment (~5.4).

The capability of agricultural mixtures to alter endpoints ranging from physiology and phenotypic expression of sex characteristics, to the more environmentally relevant reduction in reproduction, identify agricultural land and chemical use as a threat to aquatic organisms. Laboratory consequences within this study demonstrate that chemical exposure alone, not achieved in field exposures, is capable of organism and population level threats over multiple generations.

The presence of agricultural chemicals in single chemical exposures has been well documented to alter endpoints including survival (Waring & Moore, 2004), Vtg synthesis (Bringolf et al., 2004), and sex ratios (Hoskins & Boone, 2017). Yet the relative paucity of studies examining the effects of chemical mixtures makes studies such as this

important in understanding the environmental consequences of realistic mixtures. Alterations in physiology, phenotypes, and reproduction, as well as differences in biological endpoints associated with the developmental period and duration of exposure highlight the need, not only for mixture evaluations, but also total life-cycle assessments of agricultural contaminants.

Chapter 5. Conclusion

An analysis of field and laboratory fish exposure studies highlights the potential for agricultural contaminants to cause deleterious biological consequences at the molecular, physiological, organismal, and population level in two widespread North American species. Analysis of fathead minnows exposed to Maumee River waters revealed a system impacted by both agricultural and urban practices, complicated by seasonal variation. Despite the noted variations across study years, alterations in organismal functioning existed in both years indicating changes to metabolic health of exposed minnows, as well as the potential to delay and decrease total reproductive output. Caged and resident sunfish studies corroborated the fathead minnow findings, in which organisms showed significant alterations to metabolic processes, the synthesis of plasma proteins, and histological development associated with liver structure and gonad maturity.

Through the use of two species occupying discreet trophic levels, the effects of agricultural contaminants in an environmental setting indicated the potential for changes in population dynamics, as has been previously documented in field settings (Kidd et al., 2007; Palace et al., 2009). Comparison among field sites also indicates greater chemical diversity (organized for the purpose of this study into classes of use-related chemicals) at more downstream sites associated with suburban, industrial, urban, and effluent inputs. Despite greater mixture complexity at more downstream sites, apart from sunfish blood glucose, both studied species did not demonstrate

increasing severity (more detrimental to an organism's health) of endpoints at more downstream sites. The lack of correlation between increasing chemical mixture composition and concentration and biological responses at more downstream sites highlights both the complex nature of the Maumee River system and the impact which immediate surrounding land use play in affecting the biological health of aquatic organisms.

While use of a field setting impacted by agricultural practices is necessary to maintain environmental relevance in aquatic exposure experiments, the use of a laboratory multi-generational study better allowed for direct interpretation of chemical mixture effects. Impacts from co-occurring agricultural chemicals include reduced fecundity at increasing concentrations within the second exposure generation, as well as molecular and physiological alterations associated with estrogenic exposure. Synthesis of plasma proteins indicate organisms under estrogenic exposure, potentially directing energy resources away from other necessary biological functions. Further, the life-long exposure of male fish to chemical mixtures resulted in feminization as indicated by reduced expression of secondary sexual characteristics, a trait whose reduction may occlude opportunities for successful reproduction under true environmental settings (Danylchuk & Tom, 2001). Laboratory analysis of the effects of agricultural chemical mixtures on fathead minnows also highlights the need for life-cycle assessment of contaminants, as second-generation fathead minnows demonstrated greater severity in biological responses (ie. reduced SSC, increased HSI, reduced fecundity) as compared to

first-generation minnows. Differences may be due to the length and time frame of exposure (ie. larval/juvenile development, sexual differentiation) for second and third generation minnows otherwise uncaptured during the first generation.

Similar to the laboratory analysis of agricultural chemicals, resident sunfish analysis resulted in differences in biological responses unobserved in caged sunfish (ie. GSI, Vtg, gonad maturity). These differences may again be a result of exposure length and time frame as resident sunfish were present within the Maumee River for their entire life-cycle prior to analysis, a window of duration not captured during the 14-day caged sunfish deployment.

A comparison of both field and laboratory fathead minnow exposures highlights commonalities in changes to physiology and delays and reductions to fecundity. Use of both field and laboratory studies is critical in the analysis of aquatic contaminants effects on fish species, as field exposures allow for the interpretation of true environmental parameters, while laboratory analysis allow for the effects of only chemical contamination to be understood. While the use of agricultural chemicals remains necessary for current high-intensity agricultural practices (Levidow, 1998), the unintended aquatic pollution can cause both organism and population dynamic changes. These have been highlighted within the current study as indicated by changes to energy allocation, phenotypic characteristics, and reduced reproduction within organisms exposed to environmentally relevant concentrations of agricultural chemical mixtures.

The effects of agricultural contaminants on aquatic species identifies the need for shifts in agricultural practices necessary to prevent continued degradation of aquatic health. While current agricultural practices allow for the feeding of a growing global population, these same practices threaten the health of aquatic ecosystems and organisms, while also posing a risk to human health. Thus, it is necessary to shift the use of agricultural chemicals to more bio-degradable products, as well as to eliminate exposure through changes in management practices. Changes in management practices may include altering the time of year when pesticides/fertilizers are applied to avoid "wet" seasons or eliminating practices which involve extensive tilling. Changes to agricultural practices would help reduce the issue of agricultural aquatic contamination at the source.

In addition to changes necessary within agricultural practices, the added stressor of anthropogenic driven climate change may make organisms more susceptible to the threat of aquatic contamination. Adding additional stressors, such as warming water temperatures which result in lower dissolved oxygen, as well as changes to seasonal patterns, may induce further stress related responses. Through the presence of multiple stressors, climate induced changes, in combination with aquatic contaminants, may make organisms more susceptible to mortality, further harming population dynamics.

As has been shown in both chapters 3 (sunfish) and 4 (fathead minnow), the life-cycle exposure of organisms to aquatic contaminants more realistically depicts true biological consequences of exposure. Thus, there is a need within the field of aquatic

toxicology to adapt exposure protocols to better capture windows of development otherwise missed in adult-only exposures. Current 21-day exposures may not be sufficient to fully understand the effects of exposure to either single chemicals or chemical mixtures. Outcomes due to contaminant exposure will be more in line with organisms present in the environment by adjusting current protocols to include exposure of embryo, larval, juvenile, and adult life stages

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Appendix A. SOP 2.1. Maumee River MELT

MELT (Mobile Exposure Laboratory Trailer)/Trailer SOP

Objective: Successfully maintain a colony of Fathead minnows (*Pimephales promelas*-FHM) within a MELT trailer. Note: This SOP is designed for static renewal trailers only.

Necessary Supplies:

- Trailer with connected air source
- FHM feed (mixture of 2 parts brine shrimp to 1 part bloodworms)
- Jug for water exchanges
- YSI or other water sonde
- Thermometer
- Siphons/peristaltic pump
- Regular and fine mesh net

Procedure:

1. Upon arriving at the trailer each morning, perform a walk-around visual check of the trailer. Things to look for include:
    a. Locks to both trailer doors are locked.
    b. Flat tires.
    c. Any equipment left outside the trailer (ie. coolers, generators, chairs, PFDs, etc.) is locked together via cable and accounted for.
    d. General check for any visible vandalism/presence of unknown individuals near or in the trailer.
2. Morning Routine:
    a. Dilute pre-made food (1 part blood worms to 2 parts brine shrimp) with water (filtered tap water, the same used for the blank control treatment) and allow to dissolve. Constantly mix the diluted food while feeding to prevent food from settling out. Using a turkey baster, squirt approximately ¼ to 1/5 of diluted food into each aquarium.
    b. While feeding, check each aquarium for dead fish. Record dead fish on the MELT Daily Checklist Data Sheet. Usually the fish will leave the nest during feeding, making this an ideal time to check for mortality. If there is more than one aquarium from the same treatment with only one fish remaining, and the fish are the opposite sex, combine the fish into one aquarium and record in the lab notebook. It is important to keep good notes such that the number of breeding pairs is known each day when assessing fecundity later. Don't combine across treatments and don't

combine fish of the same sex just to reduce the number of aquaria in a treatment).

c. Take a YSI measurement from one aquarium in each treatment. Make sure to randomize the tanks so that different aquariums (for each treatment) are measured every day and that at least one measurement is taken from each level of the rack (the temperature at the top of the trailer may be higher than at the bottom and we want to encompass the entire range).

d. Fill the blank and positive control jugs to allow the water several hours to achieve the same temperature as the grab water samples. Prior to filling the jugs, run the hose for ~5 minutes to flush away any particulates.

i. Keep all water samples in the shade all day!

3. Early Afternoon Routine:

a. Perform the second feeding of the day. Procedure is the same as above.

4. Afternoon Routine (*Recommended to wait at least one hour after the early afternoon feeding before beginning step 3*):

a. Draw down the water in each aquarium to about half full by either turning on the spigots for each aquarium or using a siphon

i. Note that the bottom two rows of aquaria should use the spigots, while the top 3 rows should be siphoned. Do not open more than 5 spigots on the same drain tube at once as the tube may overflow due to heavy volumes of water. Use the siphon that is covered with fish netting to avoid sucking up any fish.

ii. Once all aquaria are drawn down to the half-way mark, turn all spigots to the upright position.

b. Check each aquarium for eggs and record the number of eggs on MELT Daily Checklist data sheet. To check for eggs, you can either visually assess each tile or check by gently running your finger underneath the tile to feel for the hard bumps (which indicate eggs). Ensure that the whole tile is assessed because often the FHM do not lay all of their eggs in the upper portion of the tile and will use the back or the sides as well. If no eggs are found, return the tile to the back of the aquarium on top of the rectangular grid. If eggs are present write the aquarium number on top of the tile with a pencil, count the number of eggs laid (as close to accurate as possible- if greater than 100 then within 5 is acceptable), record on the data sheet, and place the tile in a designated aquarium (one aquarium for each treatment) with only egg-containing tiles. Ensure that a large air stone is in the aquarium and is turned to medium-high to ensure proper water circulation (don't worry about having the air on too high, the better the circulation the less likely the eggs will mold over; and the eggs are

fairly well attached to the tile so they shouldn't fall off). Mark the aquarium that reproduced with a bright, colorful sticker to keep track of reproduction. Place a new tile into any aquaria that had had one removed.

c. Refill each aquarium to the fill line using the garden jug (disturb the water jugs prior to adding the water to the garden jug), making sure to pour slowly so as not to disturb the nesting tile or the fish. Always start by first filling the Control aquaria and always end with the Positive Control aquaria. Rinse the filling jug in between each treatment with filtered tap water.
d. Once all aquaria have been refilled, take a YSI measurement for each treatment (one measurement per treatment). This is most easily performed by taking a measurement from the designated hatching aquarium. Record on MELT Daily Checklist PM data sheet.
e. Perform a static renewal for the hatching aquaria in a similar manner as the exposure aquaria. Draw down the water in the designated aquarium using a siphon with a fine net to avoid sucking up larvae. Once drawn down, collect any hatched larvae and place in formalin in a labelled 1mL vial (one per clutch) for later physiological developmental analysis. Label each vial in sequential order from starting at 01. In the lab notebook, record the following info for each vial:
    i. Treatment
    ii. Parent aquarium which larvae were from
    iii. Clutch number
    iv. Date collected
    v. Number of larvae collected
f. Refill the aquarium once all larvae have been collected. Note that these hatching aquaria use the same water as the treatment aquaria.
g. Allow the fish ~10-15 minutes to calm down/resituate before performing the final feeding of the day. During this time clean up, rinse jugs with filtered water, rinse/scrub the floor of the trailer, lock all supplies together which are stored outside the trailer, and perform any other miscellaneous cleaning tasks. After cleaning up, perform feedings as described above.

Before leaving for the day, set the heater in the middle of the trailer with a fan pointed towards the ceiling. The fan will circulate the heat in the trailer and help maintain a similar water temperature from top to bottom. Ideally the AC unit and heater will be connected to a thermostat and will automatically maintain the trailer temperature between 22-25C.

Appendix B. SOP 4.1. Agricultural Multi-Generational Nest Defense

Fathead Minnow Nest Defense Analysis 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:

(In addition to aquarium containing the male FHM to be tested and a spawning tile)

Glass bowl

Mesh netting

Rubber bands

GoPro

Stopwatch

Data sheet

NOTE:

Use a stop watch when scoring tapes. Go through once in slow motion, and record all of the counts. Then go through a second time to take interaction durations (again in slow motion if needed- stopwatch won't work but you can find a duration by identifying the start and stop times on the viewer and figuring out how many seconds it is). Record times to two millisecond decimal places for precision (i.e.,2.82 seconds don't round to the whole second).

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 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 test tank mesh side down in center of target on 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 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 xcel sheet
3. Latency to 1st enter outer ring (Record the time the fish first appears within 3 cm of the jar as measured by both eyes crossing the line)
    a. Note, each square is 1cmx1cm.
4. Number of times the fish approaches within 3cm of the jar (an approach is the fishes eyes crossing the 3cm line).
5. Total duration of time the fish spends within 3cm of the jar.
6. Number of broadside (Lateral) displays
    a. The male will be parallel to the jar to show his side.
7. Latency to 1st jar bump with the snout.
8. Number of times the fish bumps the jar.
9. Duration of interaction bouts with the jar
    a. Length of time the male continuously touches the jar with his snout
    b. Single bumps receive a duration of 1 second.
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.