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**ORGANIZATIONAL EFFECTS OF DEFEMINIZING TOXICANTS: LESSONS
LEARNED FROM AN ENVIRONMENTAL SENTINEL ORGANISM,
THE FATHEAD MINNOW.**

By

Jonathan M. Ali

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Environmental Health, Occupational Health & Toxicology
Graduate Program

Under the Supervision of Professors Alan S. Kolok
and Eleanor Rogan

University of Nebraska Medical Center
Omaha, Nebraska

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Shannon L. Bartelt-Hunt, Ph.D.
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**ORGANIZATIONAL EFFECTS OF DEFEMINIZING TOXICANTS: LESSONS
LEARNED FROM AN ENVIRONMENTAL SENTINEL ORGANISM,
THE FATHEAD MINNOW.**

Jonathan M. Ali, PhD.

University of Nebraska, 2017

Supervisors: Alan S. Kolok, Ph.D. and Eleanor G. Rogan, Ph.D.

Endocrine disrupting compounds (EDCs) are chemicals that interfere with hormone function and are increasingly detected in aquatic environments, where they elicit adverse effects from exposed organisms. The toxicological effects of EDCs can be described as either activational (reversible) or organizational (irreversible), where the latter are associated with adverse outcomes in reproductive performance of adult fish. However, few studies have investigated the organizational impacts of anti-estrogenic or “defeminizing” EDCs, e.g. agrichemicals or pharmacological agents, in an environmentally-relevant or “sentinel” species. The objective of this study was to investigate the impacts of early-life EDC-initiated changes in estrogenic gene expression on organizational effects in fathead minnows. This was assessed by four experiments in which fathead minnow larvae (fhml) were exposed to defeminizing EDCs, then assessed for organizational impacts. Chapters 2 and 3 described *in situ* exposure studies conducted at the Elkhorn River Research Station to investigate the impacts of defeminizing agrichemical runoff on the gene expression and growth of fhml. Chapter 4 details the matrix-specific responses of fhml to water and sediments found within

agricultural runoff and the subsequent effects on organizational outcomes in adults, including sex ratio, sex characteristics and reproductive performance. In a subsequent study, fhml were assessed for similar organizational effects as adults following the early-life induction of estrogenic genes by exposure to the putative pharmacological anti-estrogen, fulvestrant. Early-life exposure to EDCs produced sex-specific organizational effects in fathead minnows. The early-life induction of estrogenic genes and suppression of androgenic genes contributed to organizational effects in male fathead minnows. In contrast, early-life suppression of estrogenic genes was not associated with organizational effects as adults. Taken together, the results from this study indicate that early-life exposure to defeminizing mixtures of EDCs results in organizational but not adverse impacts in the fathead minnow. The absence of adverse impacts despite the induction of organizational responses suggests that a compensatory response plays an important role in determining the adult outcomes of early-life exposures in fathead minnows.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ar	androgen receptor
ATL	Aquatic Toxicology Laboratory
cyp19a	gonadal aromatase
dmrt1	doublesex and mab-3 related transcription factor 1
dpf	days post-fertilization
dph	days post-hatch
EDC	endocrine disrupting compound
EE2	17 α -ethinylestradiol
ERRS	Elkhorn River Research Station
er α	estrogen receptor subtype α
er β	estrogen receptor subtype β
fhml	fathead minnow larvae
GSI	gonadosomatic index
igf1	insulin-like growth factor 1
K	condition factor
MS222	tricaine methanesulfonate
POCIS	polar organic chemical integrative sampler
rpl8	ribosomal protein l8
RT-qPCR	reverse transcription quantitative polymerase chain reaction
vtg	vitellogenin
WSL	Water Science Laboratory

CHAPTER 1: INTRODUCTION

Activational and Organizational Effects

The toxicological responses of an organism to a toxin can be classified based on its reversibility following removal of the chemical stimulus. Responses that are reversible and dependent on continued stimulus by a chemical are *activational*, whereas responses that are irreversible and persist after removal of a chemical stimulus are *organizational* (Guillette et al. 1995). Activational responses are immediately observable following exposure and can include up- or down-regulations of gene expression, protein translation and modification, cellular metabolism, physiological states or altered behavior (Arnold and Breedlove 1985; Guillette et al. 1995; Milnes et al. 2006). Conversely, organizational responses involve structural changes to functional motifs on DNA and chromatin (*i.e.*, epigenetic modifications), cellular differentiation patterns and tissue morphology (Guillette et al. 1995; Schäfers et al. 2007). Curiously, the susceptibility to activational and organizational impacts from chemical exposures is not uniform across an organism's life span.

In mature organisms, chemical exposures typically elicit activational responses with limited potential for subsequent adverse organizational effects. This is partially due to the establishment of fully developed metabolic pathways for the response and removal of toxic insults, as well as dosage differences due to the relative size of adults versus earlier life stages in most vertebrates. Although this does not exclude the induction of adult organizational responses such as carcinogenesis following DNA

mutation, adult organizational responses are less varied, as the fully developed organs of an adult organism present relatively fewer targets for alterations in a tissue's fate.

Chemical exposure at early life stages (e.g. embryos, neonates and juveniles) tends to produce more organizational responses relative to exposure as adults. While activational responses do occur at these early life stages, these are restricted to tissues that have differentiated and gained the cellular targets for a given toxin. The enhanced susceptibility to organizational effects is due to signaling-disruption of tissue differentiation during its window of developmental imprinting (Milnes et al. 2006; Gilbert 2014). The organizational response occurs when a sufficient dosage of a chemical either interferes with normal developmental signaling or damages key regulatory macromolecules required to maintain the normal developmental trajectory of a tissue or organ, thereby impairing its subsequent growth and function. Thus, an activational response during a critical window of development may serve as the initiator for an organizational response toward the same chemical stimulus.

Endocrine Disrupting Compounds

Endocrine disrupting compounds (EDCs), or endocrine disruptors, are a class of chemical toxins that elicit both activational and organizational responses from a diverse array of vertebrates. Endocrine disruptors affect organisms by three primary mechanisms that include: *i*) interfering with the metabolism or transport of endogenous hormones, *ii*) inhibiting the binding and signal transduction of endogenous hormones,

or *iii*) mimicking the binding properties of endogenous hormones, thereby inappropriately activating hormone receptors (NIEHS 2017). An ever-growing number of chemicals have been classified as EDCs, as continuing assessments of industrial, pharmaceutical and agricultural compounds have identified the potential for these substances to affect almost every endocrine axis (Bason and Colborn 1998; Falconer et al. 2006; Kolok and Sellin 2008). The activation and organizational effects of EDCs can be especially detrimental with respect to their capacity to dysregulate the reproductive endocrinology of vertebrates, resulting in impaired egg and sperm production, sexual differentiation and breeding success, which can culminate in population declines (Hayes et al. 2006; Kidd et al. 2007; Mathieu-Denoncourt et al. 2015; Giulivo et al. 2016).

A well-documented example of an activational effect of estrogenic EDCs in oviparous vertebrates is the induction of vitellogenin (*vtg*), an egg yolk precursor protein, following exposure to estrogen agonists (Figure 1.1). Exposure of male fish to the synthetic estrogen 17 α -ethinylestradiol (EE2) has been shown to produce circulating levels of *vtg* that are comparable to those of females in several species, including: smallmouth bass (*Micropterus dolomieu*) (Bailes et al. 2007), zebrafish (*Danio rerio*) (Martyniuk et al. 2007; Henry et al. 2009), fathead minnow (*Pimephales promelas*) (Pawlowski et al. 2004; Armstrong et al. 2016), Japanese medaka (*Oryzias latipes*) (Chikae et al. 2004), brown trout (*Salmo trutta*) (Bjerregaard et al. 2008), gold fish (*Carassius auratus*) (Martlatt et al. 2010), mummichog (*Fundulus heteroclitus*) (Chandra et al. 2012), and Murray rainbowfish (*Melanotaenia fluviatilis*) (Woods and Kumar 2011). Although structurally dissimilar from endogenous estrogens and EE2, compounds such as

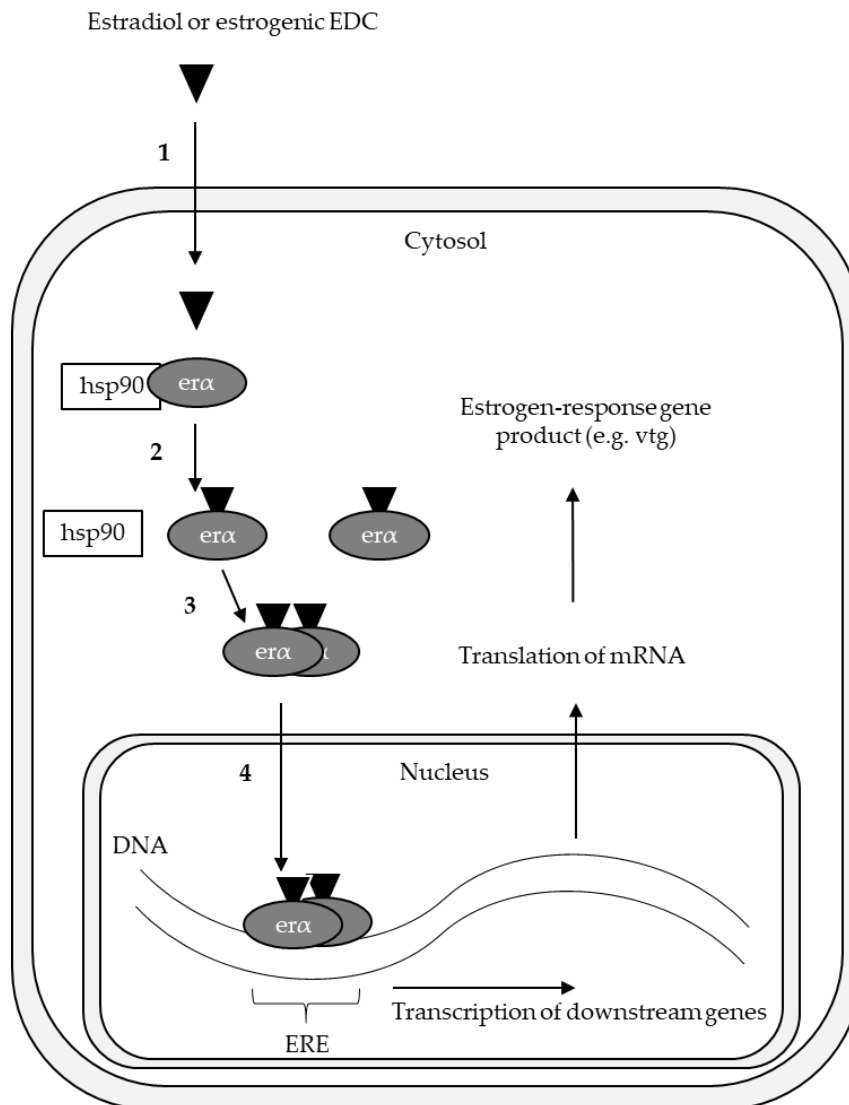


Figure 1. 1 Estrogen Signaling in Fish

Mechanism of estrogen signaling and induction of estrogen responsive gene expression at the cellular level. (1) Estradiol or EDCs with similar solubility properties diffuse across the cell membrane and bind the estrogen receptor (er). (2) Estrogen receptor-bound heat shock proteins dissociate allowing the receptor to interact with other er's. (3) Activated er's form a homodimer in the cytosol or nucleus then (4) relocates to the nucleus and binds to an estrogen response element (ERE). After binding to the ERE, transcription factors are recruited to initiate transcription of downstream estrogen-responsive genes.

plasticizers, pharmaceuticals, pesticides and phytoestrogens can also induce vitellogenin through interaction and activation of the estrogen receptor (Kortenkamp 2007; Hotchkiss et al. 2008). As an activational response, the induction of *vtg* production in male fish will cease following depuration from estrogenic EDCs (Ekman et al. 2008; Genovese et al. 2012; Baumann et al. 2014).

In addition to activational effects in adult fish, exposure to estrogenic EDCs during critical windows of development often results in adverse organizational effects. Chronic exposure to estrogenic EDCs during the window of sexual differentiation can produce organizational feminization as indicated by complete sex reversal of males, leading to female-biased sex ratios in dozens of non-gonochorist fishes (Devlin and Nagahama 2002). Gonochoristic (fixed-sex) fish rarely undergo full sex reversal but do experience “gonadal intersex”, characterized by the presence of ovarian follicle cells within the testes of genetic males, also known as “ova-testes” (reviewed by Bahamonde et al. 2013). Conversely, exposure to potent androgens, such as 17 β -trenbolone or 17 α -methyltestosterone, during sexual differentiation can result in male-biased sex ratios and impaired ovarian development, indicative of organizational masculinization (reviewed by Leet et al. 2011). Thus, the functional role of endogenous steroids in regulating sexual differentiation gives rise to a susceptibility towards organizational effects from early-life exposure to EDCs.

Extrapolating across vertebrates, this has broad implications for environmental and public health, as the conserved role of endogenous steroid hormones in regulating

sexual development and reproductive function leaves other vertebrates susceptible to the activational and organizational impacts of EDCs (Guillette et al. 1995; Diamanti-Kandarakis et al. 2009). For example, fetal exposure of mice to EE2 (0.1 µg/kg/day) or bisphenol A (10 µg/kg/day) results in the malformation of the gonadal and urinary ducts of male mice (Timms et al. 2005). In humans, epidemiological evidence suggests that early-life exposures to EDCs has adverse health impacts, such as decreased sperm quality associated with bisphenol A (Meeker et al. 2010) or the increased incidence of cryptorchidism (undescended testis) associated with pesticides (Pierik et al. 2004), without producing more drastic effects as seen in lower vertebrates (*i.e.*, sex reversal or gonadal intersex). When compared to mammals, the relatively overt responses of fishes towards EDCs allow for a more sensitive approach to detecting the subtle organizational effects of early-life endocrine disruption.

Environmental Relevance of Agrichemical EDCs

In the last several decades, a growing number of chemicals used in contemporary agricultural practices, or “agrchemicals,” have been identified as EDCs that inadvertently affect estrogen signaling in exposed fish. These agrichemicals include herbicides, fungicides, veterinary pharmaceuticals and natural steroids that are applied to crops lands and released into surface water following runoff events (Kolok and Sellin 2008; Kolok et al. 2014). In Pennsylvania, Blazer et al. (2014) found an association between the presence of such agrichemicals in water and sediments with the increased

incidence of gonadal intersex (10-100%) in smallmouth bass. More recently in Indiana, Abdel-moniem et al. (2017) found that the incidence of gonadal intersex and the induction of *vgt* expression in smallmouth bass is most prominent following spring agrichemical runoff. This evidence suggests that prolonged exposure to agrichemical EDCs elicits organizational impacts in wild populations of fish.

In Nebraska, brief exposures (7-d) to agrichemicals elicit activational responses through antagonizing estrogen-receptor mediated gene expression in sexually-mature female fish (Sellin Jeffries et al. 2011a; Sellin Jeffries et al. 2011b; Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015). Sellin Jeffries et al (2011b) found that exposure to agrichemicals that contaminate the sediments of the Elkhorn River, Nebraska, resulted in the downregulation of estrogen receptor α (*era*) expression in fathead minnows, as well as anti-estrogenic activity measured through in vitro bioassays of the human ER α . Similar reductions in hepatic expression of *era* and *vgt* have been consistently observed in female minnows following *in situ* exposure to seasonally-occurring runoff events that are predominately characterized by herbicides such as atrazine, simazine and acetochlor (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015). However, it has yet to be determined whether the same agrichemical runoff that results in the an activational defeminization of adult minnows also elicits organizational responses from earlier life stages.

Studies conducted in other Midwestern watersheds provided some limited evidence that agrichemical exposures also affect sexual development of larval fish. Leet

et al. (2012) observed male-biased sex ratios in fathead minnows that were exposed to agricultural ditchwater containing pesticides, fertilizers and steroids over the course of sexual differentiation, indicating either a masculinizing or defeminizing effect of the agrichemical mixture. Although defeminization in response to an agrichemical mixture would be consistent with the aforementioned studies in adult fish (Sellin Jeffries et al. 2011b; Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015), the omission of molecular markers for either feminization or masculinization by Leet et al. (2012) leaves the mechanism for the observed effect open to speculation. Furthermore, it is impossible to tell whether the effects were due to chronic but low levels of steroids reported by Leet et al. (2012), or the induction of organizational effects from an exposure to spring agrichemical runoff that coincided with the window of sexual differentiation. This leads to the conclusion that there is a need to investigate the activational and organizational impacts of early-life exposure to agrichemical EDCs.

Project Goals and Specific Aims

My long-term goal is to understand the role of early life exposure to xenobiotic compounds in adult health outcomes. My short-term goal and objective of my dissertation is to investigate the impact of early-life EDC-initiated induction and suppression of gene expression on organizational effects in fathead minnows. Relative to this central objective, my aims and hypotheses were:

Aim 1. To determine whether episodic exposure to EDCs in early development results in adverse organizational impacts in the fathead minnow.

Due to the fathead minnow's accepted role as an environmental sentinel organism for aquatic toxicity testing in North American watersheds (Figure 1.2; Ankely and Villeneuve 2006), several laboratory studies have been conducted to assess the impacts of EDCs on sexual development in this species (van Aerle et al. 2002; Mihaich et al. 2012; Flinders et al. 2014). However, due to the inherent difficulty and time-consuming nature of life-cycle tests using a vertebrate model, these studies have only evaluated either larvae and juvenile responses following early-life exposure (van Aerle et al. 2002; Johns et al. 2009; Leet et al. 2012; Leet et al. 2015) or the impacts of continuous lifelong exposure as adult (Länge et al. 2001; Mihaich et al. 2012; Flinders et al. 2014). Given this, the first objective of this study is to determine whether episodic early-life exposure to (anti)estrogenic-EDCs translates into organizational outcomes in the reproductive performance, sexual differentiation and sex ratios of adult fathead minnows. To accomplish this, fathead minnow larvae will be exposed to either defeminizing (Chapters 3 and 4) or feminizing (Chapter 5) conditions during sexual differentiation and assessed for subsequent organizational impacts following depuration from their original exposure conditions (Figure 1.3). It is hypothesized that episodic exposure to EDCs in early life development will result in adverse organizational impacts on adult reproductive outcomes.

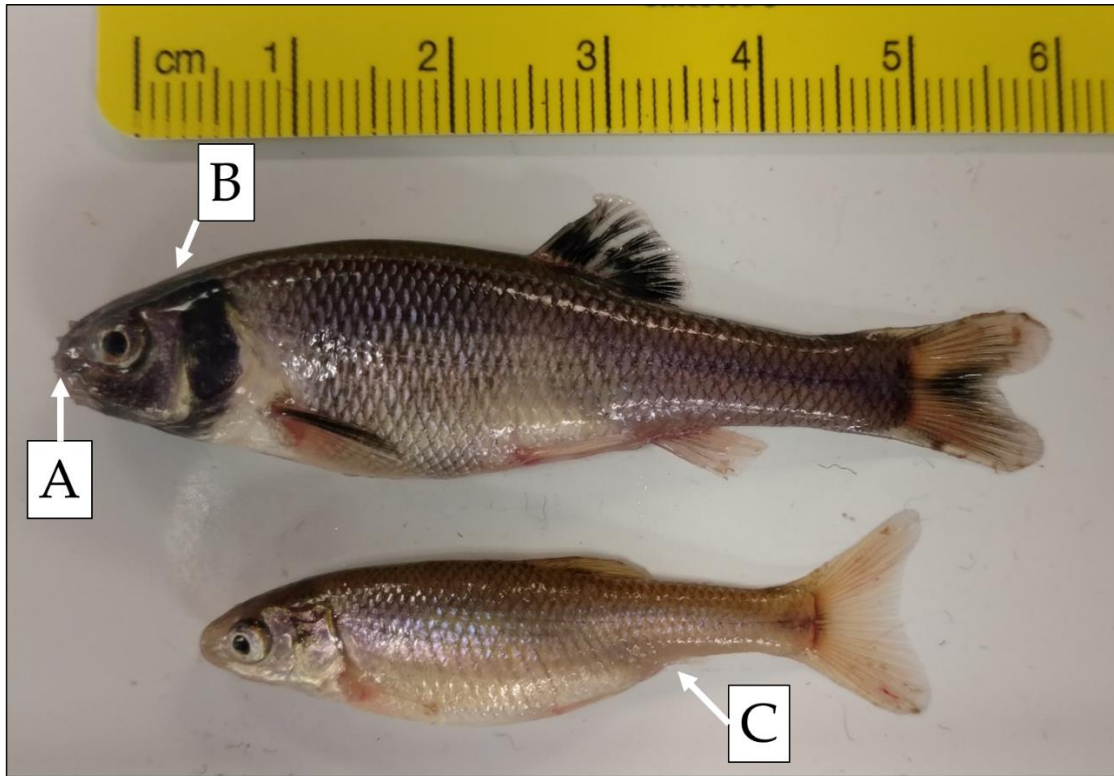


Figure 1. 2 Male and Female Fathead Minnows (*Pimephales promelas*)

Sexually mature male (top) and female (bottom) fathead minnows (*Pimephales promelas*). Fish were obtained from the Aquatic Toxicology Laboratory at the University of Nebraska at Omaha (J.M. Ali 2017). Markers of masculinization of sexually mature male fathead minnows include: nuptial tubercles (A), dorsal fat pad (B), dark coloration and larger body size relative to females. Markers of feminization of sexually mature female fathead minnows include: absence of nuptial tubercles and a fat pad, lighter coloration, and an ovipositor (C) for depositing eggs.

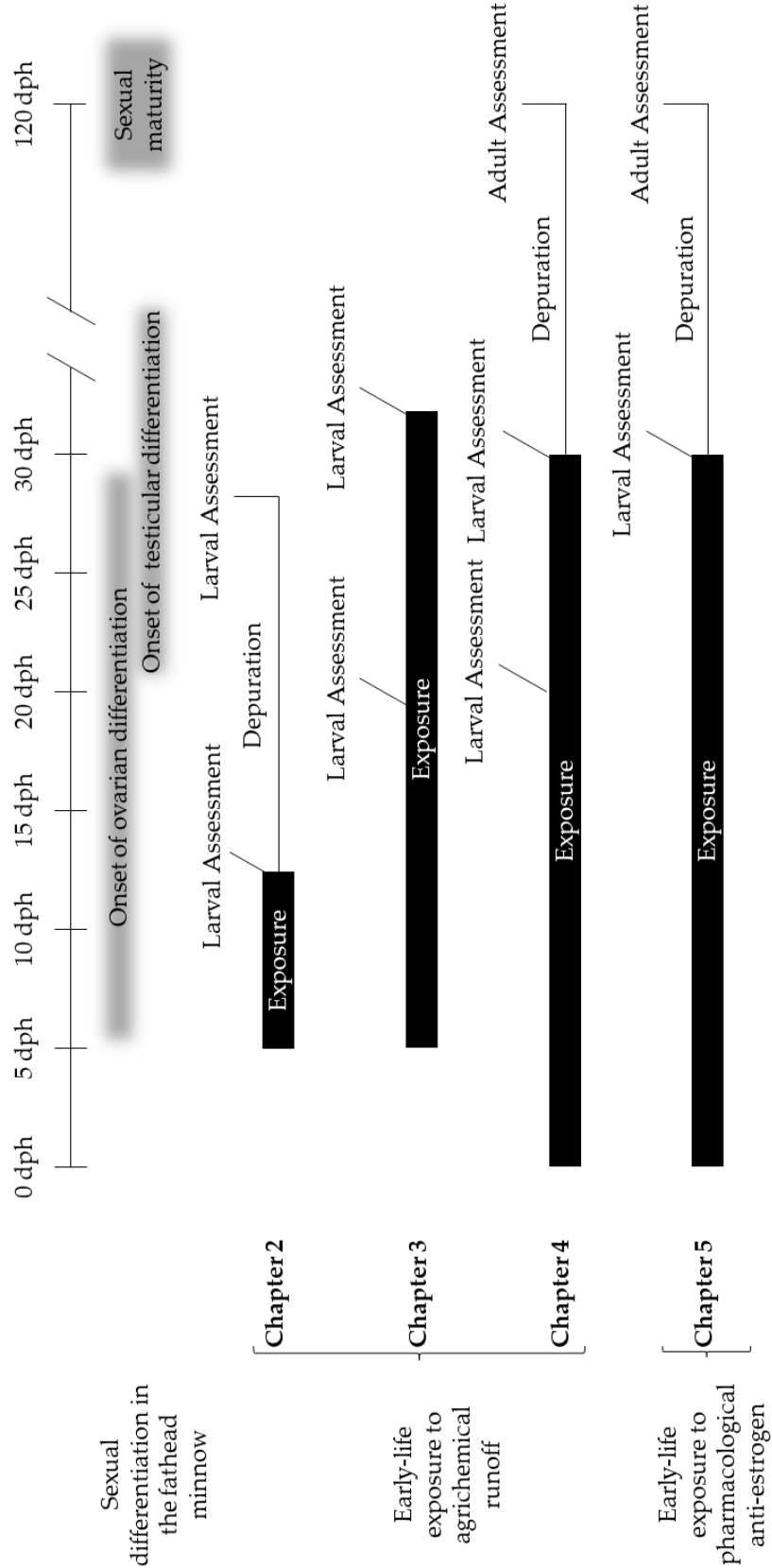


Figure 1. 3 Overview of Experiments

A comparative timeline for the experiments present in Chapters 2-5 to address the specific aims of this study. Time is indicated on the top of the figure relative to age of the fish in days-post-hatch (dph). The windows for the onset of sexual differentiation in the fathead minnow are indicated in grey, with sexual maturity for both sexes occurring at approximately 120 dph. Early-life exposure to EDCs in each experiment are shown as solid black bars, while subsequent depuration periods are shown as solid lines. Biological endpoints were collected from larval and adult fish at the times labeled as “Larval Assessment” and “Adult Assessment,” respectively.

Aim 2. Determine whether early-life suppression of estrogen-responsive gene expression in fathead minnow larvae results in adverse organizational effects

It has been established that exposure to the agrichemical mixture found in the spring runoff elicits anti-estrogenic effects in adult female fish (Sellin Jeffries et al. 2011ab; Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015). However, it has not been established whether this anti-estrogenic effect in early-life results in adverse organizational outcomes as adults. Thus, the second aim of this study is to determine whether suppression of estrogen-responsive gene expression by early-life exposure to this agrichemical mixture leads to adverse organizational impacts as adults. In Chapter 2, I outline a method for exposing minnow larvae to agrichemical runoff under *in situ* conditions at the Elkhorn River Research Station, a field station located in Eastern Nebraska, USA. In Chapters 2-4, I use a combination of *in situ* and laboratory based studies to characterize how fathead minnow larvae respond to episodic exposure to an agrichemical mixture through the changes in whole body gene expression and body size. To link these larval effects to adult outcomes, Chapter 4 describes the results from assessment of early-life exposure and subsequent adult outcomes determined by reproductive performance (*i.e.*, fecundity and fertilization success), sex characteristics and final sex ratios. It is hypothesized that episodic suppression of estrogen responsive gene expression in early life would not result in adverse organizational effects.

Aim 3. Determine whether early-life induction of estrogen-responsive gene expression in fathead minnow larvae results in adverse organizational effects.

As previously mentioned, studies in other fish species suggest that the early life effects of estrogenic EDCs result in organizational impacts on adult reproductive performance and sex ratios. Relatively fewer studies have sought to confirm whether changes in estrogen-response gene expression in fathead minnow larvae are affiliated with adverse adult outcomes as adults (Villeneuve et al. 2013), despite evidence that fathead minnows are susceptible to certain organizational effects following continuous exposure to estrogenic EDCs. Paradoxically, this will be assessed by evaluating the changes in estrogen-responsive gene expression and subsequent developmental outcomes in fathead minnow larvae following exposure to a pharmacological anti-estrogen, fulvestrant. Given the evidence from the literature, it is hypothesized that early-life induction of estrogen responsive genes would result in adverse organizational impacts.

Project Significance

The results obtained from these experiments will make a substantial contribution to our knowledge about the effects of early-life exposure to EDCs. First, these results will provide evidence as to whether isolated early-life exposures to EDCs, such as those seen during periods of agrichemical runoff, present a threat through the induction of adverse organizational outcomes on adult reproductive health. Additionally, by

broadening the framework of early life endocrine disruption beyond the traditional focus on feminization by estrogen agonists, this work will help to identify fundamental aspects of developmental toxicology in the environmental sentinel species used for environmental hazard identification and characterization. Given that the majority of early-life studies on this sentinel species rely on in-house laboratory experiments, the findings from the *in-situ* studies of Chapters 2 and 3 will play an important role in interpreting the responses of minnow larvae in the context of complex multi-stressor environments. If either early-life suppression or induction of estrogen-responsive gene expression does not result in organizational impacts, this will indicate that there are compensatory mechanisms for early-life endocrine disruption that allow fish to mitigate organizational impacts. If suppression of estrogen-responsive gene expression by agrichemicals does not result in adverse organizational impacts, this will raise questions about the specific role of estrogen signaling in the process of sexual differentiation in gonochoristic fish such as the fathead minnow. If induction of estrogen-responsive genes following an isolated early-life exposure leads to organizational impacts as adults, this will corroborate current evidence for the existing paradigm of activational and organizational effects of EDCs.

CHAPTER 2: BIOLOGICAL IMPACTS IN FATHEAD MINNOW LARVAE
FOLLOWING A 7-DAY EXPOSURE TO AGRICULTURAL RUNOFF: A MICROCOSM
STUDY.

The material in this chapter has been previously published: Ali, J. M., Farhat, Y. A., & Kolok, A. S. (2016). Biological impacts in fathead minnow larvae following a 7-day exposure to agricultural runoff: A microcosm study. *Bulletin of Environmental Contamination and Toxicology*, 96(4), 432-437. doi:10.1007/s00128-016-1762-2

Introduction

Agricultural runoff events result in the degradation of the abiotic environment and adverse health impacts on exposed aquatic organisms. Impacts on the abiotic environment include increased loading of suspended solids, nutrients, and changes in a waterbody's physiochemical parameters such as pH, temperature, conductivity and salinity (Blann et al. 2009; Kjelland et al. 2015). In addition to changes in the physicochemical characteristics of receiving surface waters, agricultural runoff contains a milieu of agrichemicals including herbicides, insecticides, fertilizers and veterinary pharmaceuticals applied to croplands that have adverse impacts on the health of downstream aquatic communities (Schulz 2004; Kolok and Sellin 2008).

The hazards of agricultural runoff towards non-target organisms have been the subject of numerous studies (reviewed in Kolok et al. 2014) particularly focusing on the toxicity of herbicides and insecticides (reviewed by Schulz 2004; Solomon et al. 2008; Udeigwe et al. 2015). Insecticide contaminants can dominate in both urban and agricultural settings throughout the summer and fall, whereas herbicides dominate runoff events in rural agricultural settings in the spring and early summer (Gilliom et al. 2006). This is evident in the Midwestern United States where the profile of agricultural runoff following spring rainfall is predominately pre- and post-emergent herbicides and suspended solids (Hyer et al 2001; Zhang et al. 2015).

Episodic exposure to agricultural runoff induces various sublethal effects in fish inhabiting agriculturally dominated waterways. Polard et al. (2011) observed an increase

in DNA strand breaks in Crucian carp (*Carassius carassius*) following a 4-d exposure to spring runoff effluent collected from an agriculturally intensive watershed in Southwestern France. In the Midwestern United States, microcosm studies utilizing female fathead minnows (*Pimephales promelas*) have observed a consistent downregulation of estrogen responsive genes, vitellogenin (*vtg*) and estrogen receptor α (*era*), following exposure to agriculture runoff (Knight et al. 2103; Ali and Kolok 2015; Zhang et al. 2015).

While studies using adult fish have identified adverse impacts following exposure to agricultural runoff (Sellin Jeffries et al. 2011; Polard et al. 2011; Knight et al. 2013; Ali and Kolok 2015), there is little knowledge regarding the effects of spring agricultural runoff on larval fish. It is important to understand the impacts of agricultural runoff on larvae because disruption of early development can result in long-term adverse outcomes realized in adult fish (Guillette et al. 1995; Villeneuve et al. 2014). The objective of this study was to investigate the impact of agricultural runoff on early-life growth and development in fathead minnow larvae.

Materials and Methods

Fathead minnows used for this experiment were obtained from the Animal Culture Unit (ACU) at the University of Nebraska at Omaha, Omaha, NE. Animals were treated in accordance with the ethical standards of the University of Nebraska Institutional Animal Care and Use Committee. Adult fish were maintained in aerated

dechlorinated Omaha tap water (laboratory water) at 25 ± 0.5 °C, pH 7.74 ± 0.28 under a 16:8 h light:dark photoperiod. Beginning on April 15, 2014, breeding triads were established consisting of one male and two females. Fish were housed in 30 L aquaria, divided into two compartments separated by a plastic, porous divider, which allowed for culturing two triads per aquarium. Breeding triads were provided with a breeding tile (12-cm-long sections of polyvinyl chloride tubing 8 cm in diameter split in half lengthwise) on which eggs were laid.

Once the triads began to breed, the tiles with adhered eggs were removed daily from the aquaria and transferred to 1 L beakers containing laboratory water maintained at 25 ± 0.5 °C. Unfertilized and fungus infected eggs were removed daily from the tiles, and surviving embryos hatched by day 5 post fertilization. Upon hatching, the larvae were transferred to 2 L beakers. A static renewal of the water within these beakers was conducted daily, replacing approximately 85% of the water with laboratory water. Once daily, all of the larvae were fed ad libitum with a mixture of 1-d-old *Artemia nauplii*. All fathead minnow larvae (fhml) were maintained under laboratory condition until 5-days post-hatch (dph), the age at the initiation of the exposure.

Exposure of 5 dph fhml was conducted at the Elkhorn River Research Station (ERRS), a research facility located immediately adjacent to the Elkhorn River in eastern Nebraska (Knight et al. 2013; Ali and Kolok 2015). The station is equipped to pump water in real-time from the Elkhorn River into microcosms capable of supporting small aquatic organisms (Kolok et al. 2012; Knight et al. 2013). Determination of the spring

runoff period at the ERRS was based upon the presence of atrazine in the Elkhorn River water during the 2014 pulse season and the occurrence of rainfall events between June 2 and 4; for details on determination of the 2014 spring pulse see Zhang et al. (2015).

The microcosms are 10 L stainless steel circular tanks maintained within a larger insulation tank (16.5 L) that circulates river water to maintain ambient water temperature (Kolok et al. 2012; Ali and Kolok 2015). While the ERRS is a protected environment, it is also an open-air facility; therefore the abiotic (temperature, photoperiod, turbidity) and biotic (planktonic community) conditions within the station microcosms are expected to be comparable to those that occur within the river. During the exposure, fhml were maintained in 3 L stainless steel chambers designed to fit inside the 10 L steel microcosms (Figure 2.1). Approximately 200 fhml were maintained within a single chamber, with one chamber used for each treatment group. A portion of the chamber was made of 86 μm stainless steel mesh, allowing river water to freely exchange between the microcosm and the larval chamber, without the loss of larvae.

Five-dph fhml were exposed for 7 days during the onset and early stages of ovarian differentiation due to evidence that this period is responsive to hormonally active compounds (Johns et al. 2009; Leet et al. 2015). Five-dph fhml for the study were randomly selected from larvae produced at the ACU (see Larvae production and maintenance) for one of two treatment groups. Station control larvae (station control) were transferred to a microcosm at the ERRS and were supplied with laboratory water daily (85% daily water change). River exposed larvae (river exposed) were placed in a

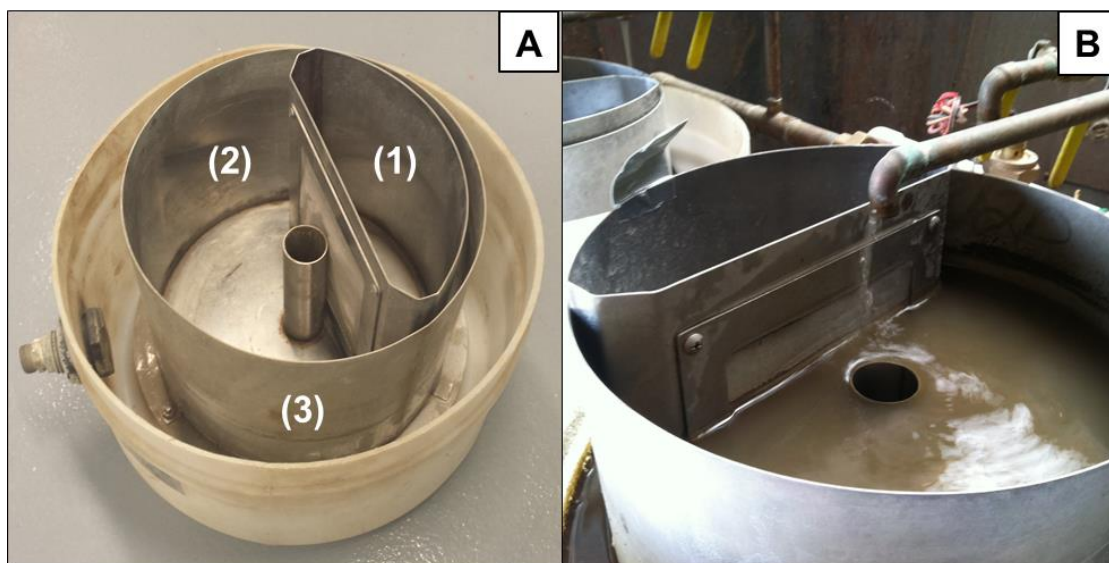


Figure 2. 1 Elkhorn River Research Station Exposure Chambers

Exposure microcosms for fathead minnow larvae (5-12 dph) maintained at the Elkhorn River Research Station. (A) Larvae were held in 3 L, half-moon shaped, stainless steel chambers (1) designed to fit inside the 10 L microcosms (2) maintained within a larger 16.5 L insulation tank (3). (B) Active microcosm receiving Elkhorn River water.

microcosm at the ERRS that received continuous water flow directly from the Elkhorn River. Both station control and river exposed microcosms were placed within insulating tubs that circulated Elkhorn River water outside of the exposure chamber to maintain similar water temperatures. Additionally, both microcosms were supplied with air-stones to control for differences in dissolved oxygen between laboratory and river water.

At the end of the 7-d exposure at the ERRS approximately 150 larvae were transferred to aerated coolers containing water from their respective microcosms and transported to the laboratory. Upon return to the laboratory from the ERRS, 15 of the 12 dph fhml were randomly sampled from each treatment group and euthanized using a lethal dose of buffered tricaine methanesulfonate (MS222), the remaining larvae were maintained in 30 L aquaria containing approximately 3 L of water and reared in laboratory water for 16 d. As the fish grew, densities of larvae within the aquaria were reduced by increasing the volume of water within the single 30 L aquarium to ensure consistent and maximal rates of growth until station control and river exposed fhml were 28 dph in age. Conditions were maintained as previously mentioned (see Larvae production and maintenance). At 28 dph, 40 fhml were randomly selected from each treatment group and euthanized using a lethal dose of buffered MS222. Body mass, length and condition factor ($((\text{total mass in mg}) / (\text{total length in mm}^3) \times 100)$) were collected from 12 dph and 28 dph fhml for morphometric analysis. Heads were removed from 12 and 28 dph fhml and bodies were flash frozen then stored at -80 °C for analysis of gene expression.

During the 7-d exposure, a series of water quality parameters, as well as chemical analysis for agrichemicals, were performed on the Elkhorn River microcosm water. The occurrence of agrichemical runoff was determined by use of atrazine test strips (Ali and Kolok 2015; Zhang et al. 2015) that detect atrazine in surface water at 3 ppb.

Temperature was continuously recorded using HOBO data loggers (Onset Corporation, Bourne, MA, USA) placed within each microcosm. Water quality measurements including pH, turbidity, dissolved oxygen and suspended solids were taken daily. Water samples were collected from water flowing into the river exposed microcosm on June 4, 6, 9, and 10 (days 1, 3, 6 and 7 of the exposure) for chemical analysis (reported by Zhang et al. 2015). In brief, two-liter grab samples of river water were collected in amber glass bottles then shipped on ice to the Water Sciences Laboratory at the University of Nebraska-Lincoln. Water samples were extracted using solid phase extraction, then analyzed for pesticides using gas chromatography mass spectrometry (GC/MS) according to previously published methods (Cassada et al. 1994).

Due to insufficient RNA yield following extraction of 12 dph larvae, gene expression analysis was restricted to 28 dph larvae. Larvae bodies were used for reverse transcription polymerase chain reaction (RT-PCR) to compare changes in the expression of gonadal aromatase (*cyp19a*), a marker gene of feminization in fhml (Leet et al. 2013). RNA extraction utilized the SV Total RNA Isolation System (Promega, Sunnyvale, CA, USA) and manufacturer's protocol to extract RNA from larval tissues. Extracted RNA was resuspended and stored in nuclease-free water at -80 °C until analysis. Extracted RNA was quantified spectrophotometrically at 260 nm by NanoDrop (NanoDrop

Technologies, Wilmington, DE, USA). Purity of the extracted RNA was assessed based on optical densities at 260 nm/280 nm and 260 nm/230 nm. Total extracted RNA samples were diluted to 10 ng· μ L⁻¹ in preparation for first-strand cDNA synthesis. Synthesis of cDNA template was performed with 0.75 μ g total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA) per the manufacturer's recommendations. All PCRs were performed using the iQ SYBR-Green Supermix (Bio-Rad) per the manufacturer's protocol. Briefly, 2 μ L of diluted cDNA template was added to 300 nM forward and reverse primers in a 15 μ L reaction volume containing iQ SYBR-Green Supermix. Expression of the target gene, *cyp19a*, was normalized with ribosomal protein (*rpl8*) as a reference gene; primer sequences were obtained from (Kolok et al. 2007; Johns et al. 2009). Normalized expression of *cyp19a* was quantified using the $2^{-\Delta Ct}$ method based on mean cycle thresholds (Ct) (Schmittgen and Livak 2008).

Sex ratio of 28 dph larval fathead minnows was determined using *cyp19a* expression as described by Leet et al. (2013, 2015). Briefly, Ct values of *cyp19a* expression were evaluated for each treatment group of larvae at 28 dph (38-40 fhml per group), separately. Distinct gaps in the Ct value at 12.5 were determined as a threshold where values less than 12.5 were treated as female and greater than 12.5 were treated as male.

Where biological parameters met the assumptions of normality and homogeneity of variance, parametric analysis was applied using JMP 9.0.1 software (SAS, Cary, NC, USA). Morphometric data for body mass, length and condition factor of station control and river exposed larvae were compared by t-test at 12 and 28 dph. At 28 dph larval

growth was compared by two-way ANOVA. Where no differences were detected by two-way ANOVA, groups were compared by treatment type, station control versus river exposed. Sex ratios were compared by Chi-square test between treatment groups. Relative expression of *cyp19a* in 28 dph female fhml was compared between treatment groups by t-test. Statistical significance was assumed at $p < 0.05$.

Results and Discussion

The objective of this study was to investigate the impact of agricultural runoff on early-life growth and development in fhml. During the 7-d exposure, the larvae were exposed to raw Elkhorn River water containing a very high sediment load, as well as a suite of herbicides, (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015). Furthermore, the larvae were exposed on site, meaning that they also experienced an environmentally relevant photoperiod and daily oscillations in water temperatures.

At 28 dph, the fhml that were exposed to agricultural runoff experienced a significant reduction in the relative gene expression of *cyp19a* compared to station controls (Figure 2.2; t-test, $p < 0.05$). This result is consistent with previous studies (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015) in which adult fathead minnows were maintained at the station for 7 d under similar environmental conditions. In 2012, Knight et al. (2013) demonstrated that elevated agrichemical concentrations in the Elkhorn River led to the defeminization of *vtg* and *era* expression in adult female minnows whereas exposure to river water following the spring runoff had no impact of estrogen

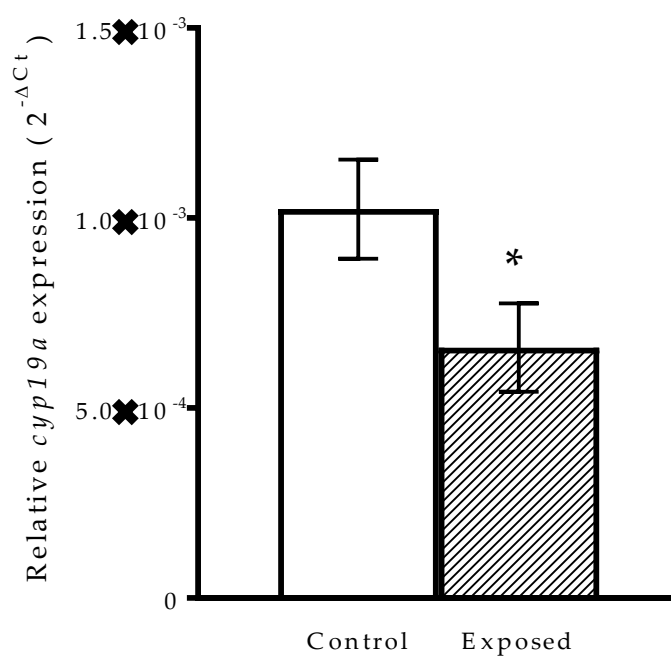


Figure 2. 2 Gene Expression Analysis for 2014 Minnow Larvae

Relative gene expression of gonadal aromatase (*cyp19a*) for station control and river exposed female fathead minnow larvae at 28 dph. Values presented as mean \pm SEM (n=12-16). Significant differences denoted by asterisks (t-test; $\alpha=0.05$).

Table 2. 1 Physicochemical Parameters of the 2014 Exposure

Physical and chemical parameters for water within the station control and river exposed microcosm maintained at the Elkhorn River Research Station from June 4-11, 2014. Values presented as the mean (\pm SD) over the seven-day exposure period (n=7).

	Temperature (°C)	pH	DO (%)	Turbidity (NTU)	Suspended solids (mg·L ⁻¹)	In flow (mL·min ⁻¹)	
Station control	22.1 (±1.4)	7.23 (±0.16)	77.0 (±17.5)	68.4 (±14.0)	220.3 (±12.6)	-	
River exposed	21.1 (±2.1)	7.93 (±0.10)	84.7 (±9.3)	993.0 (±5.0)	4,507 (±3,370)	946 (±801)	
Date							
Pesticides (µg·L ⁻¹)*	June 4	June 5	June 6	June 7	June 8	June 9	June 10
Acetochlor	0.21	-	4.77	-	-	2.53	1.61
Atrazine	1.35	-	6.27	-	-	5.30	4.75
Deethyatrazine	0.22	-	1.12	-	-	0.92	0.84
Deisopropylatrazine	0.27	-	0.67	-	-	0.50	0.51
Dimethenamid	0.06	-	0.38	-	-	0.33	0.24
Metolachlor	0.25	-	1.43	-	-	1.58	1.11
Propazine	0.03	-	0.12	-	-	0.09	0.08
Simazine	0.01	-	0.02	-	-	0.02	0.01

*Reported by Zhang et al. (2015)

responsive genes. More recently, serial 7-d exposures of adult minnows to runoff in the Elkhorn River has associated the molecular defeminization of female fish with major discharge events carrying pesticide-laden sediments (Ali and Kolok 2015; Zhang et al. 2015).

A major difference between the current study and previous studies that featured adult minnows was the timing of gene expression analysis. In previous studies, adult minnows were sacrificed immediately after the field exposure. In the current study, a subset of larvae were sacrificed immediately after the exposure (15 dph) however insufficient RNA yield precluded gene expression analysis. Surprisingly, when the larvae were given 16 additional days to grow (under controlled laboratory conditions) significant differences remained in their relative gene expression of *cyp19a*. This result suggests that the response of the larvae may not have been purely a result of immediate exposure, but rather that changes in the environment the larvae have been exposed to translated to implications at later developmental stages.

In the present study, the fhml that were exposed to Elkhorn River water were smaller, in both mass and body length, than the station controls (Table 2.2). Consistent with the results from the gene expression analysis, the larvae at 12 dph, showed no differences in body mass or total length, however, 28 dph river exposed fhml were significantly smaller in length and body mass than their 28 dph station control counterparts (t-test, $p < 0.01$). This result suggests that the river exposed fish experienced changes due to exposure that were realized days after the exposure ended.

Table 2. 2 Morphometric Analysis for 2014 Minnow Larvae

Body mass, length and condition factor of fathead minnow larvae from station control and river exposed treatment groups at 12 and 28 dph. Values presented as mean \pm SEM. Asterisks denote significant differences relative to station control following t-test ($\alpha=0.05$).

Age (dph)	Treatment Group	n	Body Mass (mg)	Body Length (mm)	Body Condition Factor
12	Station control	15	5.8 \pm 0.4	8.6 \pm 0.2	0.91 \pm 0.07
	River exposed	15	4.7 \pm 0.4	8.1 \pm 0.2	0.86 \pm 0.07
28	Station control	40	149.6 \pm 6.3	17.4 \pm 0.3	2.81 \pm 0.09
	River exposed	40	122.8 \pm 6.3*	15.9 \pm 0.3*	2.93 \pm 0.13

There is at least one other study in which fhml have been exposed to agricultural waters under semi-natural field conditions and showed changes in the response of growth. Leet et al. (2012) documented increased body mass and length in fhml following 40 to 45-day exposure to whole agricultural ditch water. Such changes in growth can be attributed to hormonally active chemicals found in agricultural runoff, but this was confounded by warmer water temperatures of ditch water exposed larvae (26 ± 2 °C) with respect to their laboratory water controls (24 ± 1 °C) (Leet et al. 2012). Furthermore, these results lacked a molecular marker to associate altered hormonal function with changes in growth following exposure to agricultural runoff.

Despite the differences in size between the station exposed and station control fish, the 28 dph larvae were not different in condition factor. Condition factor is used to indicate the relative condition (health) of fish, and the absence of a difference between these two groups implies that the overall health of the two groups is consistent. It is probable that the smaller mass and length of the river exposed fish is indicative of a slower growth rate, but not a reduction in overall health.

The sensitivity of larval fathead minnows to environmental stressors observed in growth and gene expression merit further investigation into the biological impacts of episodic runoff events to determine whether early-life impacts translate into adverse adult outcomes. Future studies would benefit by combining laboratory and microcosm-based approaches to account for the effects of certain non-chemical abiotic factors, and by prolonging exposure duration to explore long-term impacts of agricultural runoff

throughout sexual development. While the long-term impacts remain unclear, in situ exposures similar to the present study are promising to understand impacts of agricultural runoff on growth, development and reproductive success in wild populations.

CHAPTER 3: COMPENSATORY RESPONSE OF FATHEAD MINNOW LARVAE
FOLLOWING A PULSED *IN-SITU* EXPOSURE TO A SEASONAL AGRICULTURAL
RUNOFF EVENT.

The material in this chapter has been previously published: Ali, J. M., Sangster, J. L., Snow, D. D., Bartelt-Hunt, S. L., & Kolok, A. S. (2017). Compensatory response of fathead minnow larvae following a pulsed in-situ exposure to a seasonal agricultural runoff event. *Science of the Total Environment*, 603-604, 817-826.
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Introduction

Successful development of any organism requires an accommodating environment. This is especially true for aquatic vertebrates such as fish that rely on a combination of environmental and genetic cues to regulate early life growth and ontogeny (Baroiller 2009; Pittman et al. 2013). Diel and seasonal oscillations in abiotic factors such as temperature, dissolved oxygen and suspended solids can profoundly alter growth, metabolism and survival of fish larvae (Pérez-Domínguez et al. 2006; Pérez-Domínguez and Holt 2006; Shrimpton et al. 2007; Villamizar et al. 2012; Armstrong et al. 2013). Beyond growth and development, several abiotic factors are also recognized to influence sexual determination and differentiation in a wide variety of species (as reviewed by Devlin and Nagahama 2002). For example, exposure of West African cichlid larvae (*Pelvicachromis pulcher*) to slightly acidic conditions (pH 5.5) during early development results in a female-biased sex ratio (Reddon and Hurd 2013). Although fish acclimate to natural variation in their native environments, the presence of anthropogenic stressors presents novel challenges for larval and juvenile fish.

Exposure to agricultural runoff is one such example of a widespread anthropogenic stressor that can influence larval fish. In many Midwestern streams, agrichemical concentration is seasonal with the highest concentrations occurring during the spring (Kolok et al. 2014). As a pollutant mixture, agricultural runoff contains fertilizers (Kaushal et al. 2011), pesticides (Schulz 2004; Vecchia et al. 2009; Lerch et al. 2011a), and veterinary pharmaceuticals (Kolok and Sellin 2008; Biswas et al. 2013;

Jaimes-Correa et al. 2015) which move downstream as “pulses” that persist on an order of days to weeks depending on the hydrology of the affected watershed (Blann et al. 2009). Throughout the spring there will be a series of short-term pulsatile events which in composite will make up the overall spring pulse (Ali and Kolok 2015). The short-term peaks in agrichemicals tend to overlap with dramatic fluctuations in physicochemical parameters (e.g. temperature, dissolved oxygen, salinity, suspended solids) related to increased river discharge (Blann et al. 2009; Zhang et al. 2015). Early life stage fish may be particularly sensitive to the covariation among stressors, as larvae are balancing the metabolic demands of growth and organogenesis while simultaneously responding to their unpredictable surroundings.

In the Midwestern United States, agrichemical pulses follow precipitation events from May until July (Crawford 2001; Smiley et al. 2014; Zhang et al. 2015), making a predictable and natural setting for investigating their biological impacts. Indeed, field studies exposing fish to seasonally occurring agricultural runoff have documented endocrine disruption in the reproductive axis of otherwise intact adult females (Sellin et al. 2009; Sellin Jeffries et al. 2011a; Sellin Jeffries et al. 2011b; Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015). Periods of elevated discharge and pesticide loads were associated with decreased expression of the steroid responsive genes vitellogenin (*vtg*), estrogen receptor subtype α (*era*) and androgen receptor (*ar*) (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015).

In larval fish, steroid receptors regulate gene expression, growth and organogenesis. Leet et al. (2012) found that fathead minnow larvae (*Pimephales promelas*) (0-45 days post fertilization), exposed to agricultural ditch water contaminated with pesticides and androgenic steroids under semi-natural conditions had increased body masses and a male biased sex ratio relative to lab water controls. More recently, Ali et al. (2016) reported that the brief *in situ* exposure of fathead minnow larvae, 5-12 days post hatch (dph), to an agrichemical pulse resulted in impaired growth and persistent suppression of the steroidogenic enzyme aromatase despite a 16-d recovery period in clean water.

Developmental plasticity presents a major challenge for understanding the response of larval fish towards episodic stressors. Plasticity allows the larvae to match its rate of development to oscillations within the environment (Pittman et al. 2013). Under adverse conditions development may be attenuated. When optimal conditions are restored, development may compensate, returning to a normal trajectory (Ali et al. 2003). Following a simulated cold front, red drum larvae (*Sciaenops ocellatus*) reared under diel thermocycles exhibited enhanced growth and feeding relative to larvae reared at constant temperatures (Pérez Dominguez et al. 2006). While thermal effects on developmental depression and compensation have been well studied, there is a paucity of literature that highlights the influence of weather-driven episodic exposure to physicochemical stressors from agricultural sources.

To date, there are very few studies that utilize larval fish for *in situ* exposures, and even fewer that investigate the impact of an episodic stressor like agricultural runoff. Furthermore, many of these studies only examine biological endpoints at a single time point after the exposure, an experimental design that fails to characterize how larval plasticity responds to intermittently polluted environments. The objective of this study is to characterize the developmental plasticity of fathead minnow larvae in a natural environment subject to a seasonal episodic perturbation in the form of a complex mixture of agricultural stressors. We hypothesized that 1) larval fish subjected to an agrichemical pulse under natural conditions would experience down regulations in endocrine function and growth immediately following exposure, and 2) the exposed larvae would show partial or complete compensation in endocrine function and growth following a recovery period in the field. To assess this, fathead minnow larvae were maintained at the Elkhorn River Research Station over the course of an agricultural runoff event. Larvae were assessed for changes in endocrine responsive gene markers and growth after 14 and 28 d following the start of the exposure and compared to controls maintained in clean water at the Elkhorn River Research Station.

Materials and Methods

Animal production and maintenance

Fathead minnow larvae (*Pimephales promelas*) used for this experiment were obtained from the Animal Culture Unit at the University of Nebraska at Omaha, Omaha,

NE. All procedures were conducted in compliance with protocols approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (Protocol #98-075-110). All fish were maintained in dechlorinated tap water at 25 ± 1.0 °C. Beginning on April 15, 2015, breeding triads of adult fathead minnows were established consisting of one male and two females. Fish were housed in 30 L aquaria, divided into two compartments by a plastic, porous divider with two triads in each aquarium. Breeding triads were provided with a breeding tile (12-cm-long sections of polyvinyl chloride tubing 8 cm in diameter split in half lengthwise) on which eggs were laid.

After the triads began to breed, the tiles with eggs were removed daily from the aquaria and transferred to 1 L aerated beakers. Unfertilized and fungus infected eggs were removed daily, and surviving embryos all hatched by 5 days post fertilization. Upon hatching, the larvae were transferred to 1 L beakers at a density of 100 larvae per liter. A daily static renewal of the water within these beakers was conducted replacing approximately 80% of the total volume. All larvae were fed daily with a mixture of newly hatched (<24 h old) *Artemia nauplii* (INVE Aquaculture, Salt Lake City, UT).

Exposure at the Elkhorn River Research Station

Exposure of all fathead minnow larvae was conducted at the Elkhorn River Research Station (ERRS), where previous field studies have identified biological impacts and changes in endocrine responsive gene expression following *in situ* exposure to

agricultural runoff (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015; Ali et al. 2016). The ERRS is an open-air facility located approximately 10 km upstream from the confluence of the Elkhorn and Platte Rivers, Nebraska, USA. The station is equipped to pump water continuously from the Elkhorn River into stainless steel mesocosms capable of supporting aquatic organisms (Ali and Kolok 2015; Zhang et al. 2015; Ali et al. 2016).

The sampling regime for water, sediment and biological samples is outlined in Figure 3.1A. Biological sampling consisted of a subset of fish taken 14-d after the start of the exposure (Exposure 1) to evaluate the immediate impact of the pulse, while the remaining fish were sampled 14 d after the pulse had subsided to evaluate their post-pulse recovery (Exposure 2). Passive samplers (POCIS) were deployed over three distinct 14-d windows (i.e. Pre-Pulse, Pulse and Post-Pulse) (see 2.4 Water and sediment sampling) to evaluate changes in aqueous agrichemical contamination. Finally, weekly sediment samples (S) were collected across the larval fish exposure to quantify the overall contribution of sediment-associated agrichemicals to the *in-situ* exposure.

The start of the agrichemical pulse was determined using atrazine test strips (Abraxis, Warminster, PA) as described in previous studies within the Elkhorn River watershed (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015). Briefly, beginning April 15, 2015 atrazine test strips were used to test whether concentrations of atrazine in surface water in the Elkhorn River were above or below $3 \mu\text{g}\cdot\text{L}^{-1}$. These test strips have been demonstrated to accurately detect the presence of atrazine and other triazine herbicides at $3 \mu\text{g}\cdot\text{L}^{-1}$ in surface water (US EPA 2004), with further confirmation of there

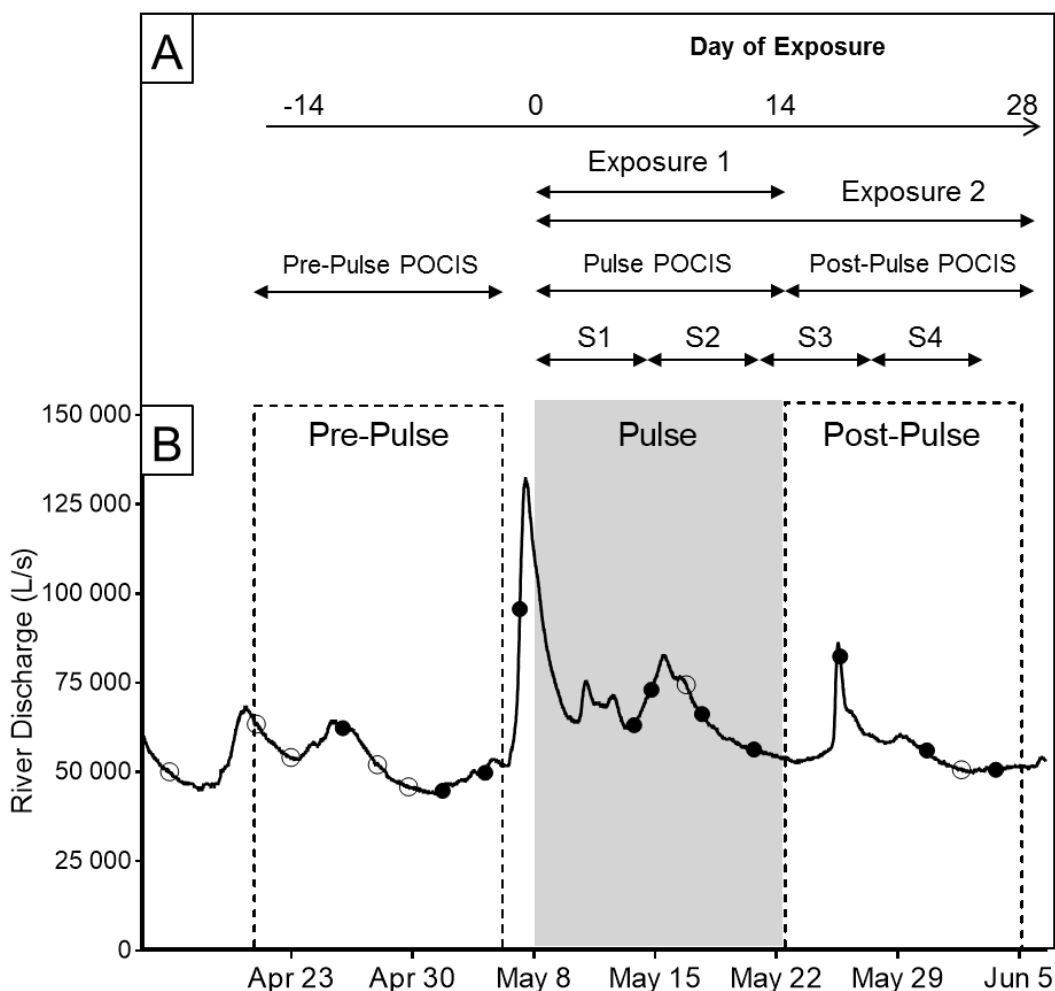


Figure 3. 1 Experimental Design for the 2015 Field Exposure

Experimental design (A) for exposure of fathead minnow larvae at the Elkhorn River Research Station above corresponding temporal changes in Elkhorn River discharge (B). Exposure periods for fathead minnow larvae (Exposures 1 and 2), water sampling periods by polar organic chemical integrative samplers (POCIS), and sediment collection periods indicated by labeled lines. The temporal changes in discharge were based on data collected from the USGS Elkhorn River (06800500) gauging station at Waterloo, Nebraska, USA. Detection of the agrichemical pulse conducted using atrazine test strips (see Methods). Positive atrazine test strip results are shown as solid circles, and negative test results are represented as open circles. Pre-pulse, Pulse and Post-Pulse periods indicated by boxes.

accuracy by previously collected field data (Knight et al. 2013; Ali and Kolok 2015). Tests were conducted every three to four days where the detection of three consecutive positive atrazine strips determined the start of the spring agrichemical pulse on May 8, 2015 (Figure 3.1B). After the deployment of fathead minnow larvae, atrazine strip tests were conducted weekly and following rainfall events.

Fathead minnow larvae, 5 days post-hatch (dph) were deployed from May 8 until June 5, 2015 and sampled at the end of Exposure 1 (19 dph) and Exposure 2 (33 dph) (Figure 3.1A). During the exposure, larvae treatment groups were maintained at the ERRS in 3 L, half-crescent shaped, stainless steel chambers designed to fit inside the 10 L mesocosms (Ali et al. 2016). A portion of the 3 L chamber was made of 86 μm stainless steel mesh, allowing river water to flow freely between the mesocosm and the larval chamber, without the loss of larvae. The 10 L mesocosms were maintained within a larger insulation tank (16.5 L) that circulated river water to maintain ambient water temperature of the Elkhorn River.

Two treatment groups, station control and river exposed, were maintained in separate mesocosms at the ERRS for a 28-day exposure (5-33 dph). One hundred larvae were transferred into each mesocosm at the start of the exposure. Station control larvae were transferred to a mesocosm at the ERRS that was supplied with dechlorinated tap water replacing the total volume by 80% daily. River exposed larvae were placed in a mesocosm at the ERRS that received continuous water flow directly from the Elkhorn River. Both treatment groups were fed, ad libitum, newly hatched *A. nauplii* once daily.

Both station controls and river exposed mesocosms were placed within insulating tubs that circulated Elkhorn River water outside of the exposure chamber to maintain similar water temperatures. Additionally, both mesocosms were supplied with air-stones to control for differences in dissolved oxygen between treatment groups.

At the end of Exposure 1 and Exposure 2, larvae were transferred to aerated coolers containing water from their respective mesocosms and transported to the laboratory. Larvae from each treatment group at the end of Exposure 1 (n=35-36) and Exposure 2 (n=20) were euthanized using a lethal dose of MS222. Body mass and length were collected from both age groups for morphometric analysis. Whole larvae were flash frozen in liquid nitrogen, then stored at -80 °C for analysis of gene expression by RT-qPCR.

Water and sediment sampling

Water quality measurements, including temperature (°C), pH, conductivity ($\mu\text{S}\cdot\text{cm}^{-1}$), dissolved oxygen ($\text{mg}\cdot\text{L}^{-1}$) and suspended solids ($\text{mg}\cdot\text{L}^{-1}$) in both station control and river exposed mesocosms, were taken daily. Sampling for aqueous pesticides was conducted using polar organic chemical integrative samplers (POCIS) (Environmental Sampling Technologies, St. Joseph, MO, USA). Prior to deployment, all POCIS were soaked in 2 L Nanopure water ($19.7\text{ M}\Omega$) for 24 hours. A single POCIS was deployed within the receiving 10-L mesocosm for each of the three sampling periods; a Pre-pulse (April 21-May 5), Pulse (May 8-22), and Post-pulse (May 22-June 5). A

laboratory blank was maintained in dechlorinated laboratory tap water for 14 d to evaluate the laboratory water used for the station control mesocosm. At the end of deployment POCIS were stored separately at -20 °C until further analysis.

Suspended solids carried in the Elkhorn River accumulated as shoals of sediment in the bottom of the larval mesocosms over the course of the 28-d exposure. Sediment was collected weekly from these shoals for physical and chemical analysis. A composite sample was collected weekly using glass jars and frozen at -20 °C for pesticide analysis by the Water Sciences Laboratory at University of Nebraska Lincoln (described below). The remaining bulk sample was stored at room temperature for approximately 48 hours allowing suspended solids to settle before removing excess water. The remaining sediment was dried at 100 °C until a constant mass was reached (12-24 h). Dry sediment samples were analyzed using standard methods to determine texture (Gee and Or 2002) and total organic carbon (TOC) (Islam et al. 1998).

Water and sediment chemistry

Sediment and POCIS samples were stored at -20 °C. Sediments were processed using microwave assisted solvent extraction (MASE) and extracts were analyzed using gas chromatography-mass spectrometry (GC/MS) Zhang et al. (2015). POCIS were extracted according to previously published protocols Sellin et al. (2009). Reference compounds and high purity solvents (Optima, Fisher Scientific) were obtained from Thermofisher (St. Louis, MO) or Sigma-Aldrich (St. Louis, MO). Labelled internal

standards $^{13}\text{C}_3$ -atrazine, $^{13}\text{C}_3$ -deethylatrazine, and $^{13}\text{C}_3$ -deisopropylatrazine were obtained from Cambridge Isotopes (Tewksbury, MA). A complete list of target compounds is included in Supplemental Table 1. Surrogate compounds, terbuthylazine and butachlor, were added at the beginning of sample processing to help quantify losses of chemically similar target compounds, while internal standards were added near the end of processing for calibration of the instrument response for each compound as described below.

A suite of pesticides was selected for measurement due to regional application practices and their regular detection during the spring pulse in previous studies within the Elkhorn River watershed (Ali and Kolok 2015; Zhang et al. 2015). Included in this suite of pesticides were atrazine, acetochlor, metolachlor all of which are consistently detected within spring to early summer runoff (Lerch et al. 2011a; Lerch et al. 2011b; Knight et al. 2013; Ali and Kolok 2015; Fairbairn et al. 2016).

Extraction of POCIS followed published protocols Alvarez (2004) and Kolok et al. (2014, Sellin et al. (2009)). Briefly, samplers were rinsed with reagent water, disassembled, membranes separated and sorbent material quantitatively transferred using ~10 mL of methanol to glass chromatography columns packed with a small plug of glass wool. Target compounds are then slowly eluted from the POCIS sorbent using 50 mL of a 1:9 mixture of methanol and ethyl acetate into glass evaporation tubes (RapidVap, Labconco, Kansas City, MO). The extract was spiked with 2,000 ng of surrogate compounds (terbuthylazine and butachlor), evaporated by heating and

vortexing under a nitrogen stream at 50 °C until 2-3 mL solvent remained. The concentrated extract was transferred to a glass culture tube and mixed with anhydrous sodium sulfate to remove residual water. The dried extract was quantitatively transferred to a second culture tube with additional ethyl acetate, spiked with 5,000 ng labelled internal standards and evaporated to approximately 100 µL and then transferred to a 2 mL autosampler vial fitted with a 300 µL silanized glass insert using ~200 µL of ethyl acetate. Converting the instrument detection limits (IDL=3s) based on the variability of the lowest standard (250 ng·mL⁻¹), the overall detection limits in the POCIS are conservatively estimated to be near 20 picograms (pg) on column corresponds to 5 ng recovered from the POCIS using a 250 µL final extract volume. Surrogate recovery averaged $52 \pm 26\%$ in POCIS extracts. A fortified blank was prepared by spiking 1,000 ng of analyte into the evaporation tube and analyzed as a sample. Target compound recovery averaged $77 \pm 40\%$. A laboratory reagent blank containing only surrogate and internal standard compounds contained no analyte above the estimated detection limit.

Sediment samples were extracted using MASE techniques with a MARS XPress (CEM Corporation, Matthews, NC) microwave system. Briefly, 5.0 grams of thawed sediment was accurately weighed into a 10 mL Teflon™ microwave tube, mixed with 6 mL of acetonitrile and spiked with 400 ng of surrogate compounds. Samples were mixed by vortexing, and then microwaved at 800 W temperature ramped to 90 °C over 10 minutes and held at 90 °C for 5 min. After cooling to room temperature and allowing particles to settle, the acetonitrile was transferred to glass evaporation tubes (RapidVap

N₂, Labconco, Kansas City, KS). An additional 10 mL of acetonitrile was added to the sediment to effect quantitative transfer and mixed by vortexing for 30 s. After settling, the second portion of acetonitrile combined in the evaporation tube and concentrated under nitrogen at 45 °C until ~1-2 mL of extract remained. The concentrated extract was transferred to a glass culture tube, spiked with 1000 ng labelled internal standard, any residual water removed by pipetting, and then dried with anhydrous sodium sulfate. The extracted sample was then transferred to a second culture tube using ethyl acetate, evaporated to 100 µL, and then transferred to a 2 mL autosampler vial fitted with a 300 µL silanized glass insert using ~200 µL of ethyl acetate. Method detection limits (MDL) were determined by extraction of 8 replicates of sand spiked at 4 ng·g⁻¹ (Supplemental Table 1).

All extracts were analyzed on an Agilent 5973 GC/MS outfitted with a Leap CombiPAL autosampler with split-less injection using a Restek (Bellefonte, PA) Rtx-1, 30m x 0.25 mm ID and 0.25 µm film thickness capillary column. Oven temperature was programmed to run at 80 °C for 0.75 min, ramp to 170°C at 40°C·min⁻¹, ramp to 236 °C at 2.5 °C·min⁻¹, ramp to 275 °C at 40 °C·min⁻¹ and hold for 9.62 min. The injection port temperature was 250 °C and the transfer line interface temperature was 280 °C. Retention times, quantifying ions, and instrument detection limits determined from repeated analysis of the lowest standard are included in Supplemental Table 1. ¹³C₃-atrazine was used as the internal standard for all compounds except for DEA and DIA which used their respective labelled analogues.

Relative gene expression analysis

Whole larvae bodies were used for reverse transcription quantitative polymerase chain reaction (RT-qPCR). RNA extraction utilized the SV Total RNA Isolation System (Promega, Sunnyvale, CA, USA) following manufacturer's recommendations. RNA was resuspended and stored in nuclease-free water at -80 °C until analysis. Purity and concentration of RNA were assessed by Nanodrop (NanoDrop Technologies, Wilmington, DE, USA) based on optical densities at 260 nm/280 nm and 260 nm/230 nm. Total extracted RNA samples were diluted to 15 ng·μL⁻¹ in preparation for cDNA synthesis. First-strand cDNA synthesis was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA) per the manufacturer's recommendations. All PCR reactions were performed on the CFX Connect Real-Time PCR Detection System using the iTaq Universal SYBR® Green Supermix, 2x concentration, (Bio-Rad) per the manufacturer's protocol. Briefly, 2 μL of diluted cDNA template was added to 20 μM forward and reverse primers in a 15-μL volume containing iTaq Supermix. Target genes involved in steroid signaling and synthesis were selected for analysis (primer sequences and their sources are provided in Supplemental Table 2), these included: androgen receptor (*ar*), insulin-like growth factor 1 (*igf1*), 17β-hydroxysteroid dehydrogenase (*hsd17b*), gonadal aromatase (*cyp19a*), doublesex and mab-3 related transcription factor 1 (*dmrt1*) and estrogen receptor subtype 1 (*era*). The reference gene ribosomal protein (*rpl8*) was used to normalize gene expression (Kolok et al. 2007). All reaction efficiencies were between 85 and 110%.

Statistical analysis

Data were analyzed using JMP 11 software (SAS, Cary, NC, USA). Morphometric data for Exposure 1 (n=35-36) and Exposure 2 (n=35-36) larvae were compared between treatment groups using t-test. Comparison of relative gene expression between station control and river exposed larvae from Exposure 1 (n=10-12) and Exposure 2 (n=16-20) was also conducted using t-test. Welch's t-test was used when the assumption of equal variances between treatment groups was not satisfied as determined by Bartlett's test. Statistical significance was assumed at $p < 0.05$.

Results

Water quality and chemistry

The 2015 spring pulse, as determined by atrazine testing, began in early May and coincided with an approximately 2.5-fold increase in Elkhorn River discharge (Figure 3.1). Over the course of the 28-d sampling period there was a major discharge event that peaked on May 8, followed by two lesser peaks in river discharge on May 15 and May 26. There was considerable fluctuation in the levels of suspended solids being transported within the Elkhorn River which coincided with periods of elevated discharge.

Within the mesocosms, fathead minnow larvae experienced variation in water quality parameters based on water source (i.e. laboratory or river water). Average values (\pm SD) for water quality parameters in the river exposed mesocosm are summarized in

Table 3.1. Station control larvae experienced less variation in water pH (7.52 ± 0.32), conductivity ($457.1 \pm 39.8 \mu\text{S}\cdot\text{cm}^{-1}$), and suspended solids ($240.1 \pm 184.4 \text{ mg}\cdot\text{L}^{-1}$) than did the river exposed fish, but larvae in both groups were exposed to natural diel oscillations in water temperature (Figure 3.2). Changes in dissolved oxygen levels of station control larvae decreased from $7.78 (\pm 1.15) \text{ mg}\cdot\text{L}^{-1}$ over the pulse period to $5.86 (\pm 0.95) \text{ mg}\cdot\text{L}^{-1}$ as temperature increased over the post-pulse period which were comparable to variations measured in the river exposed mesocosm (Table 3.1).

Analytical chemistry data collected from POCIS confirmed the elevated agrichemical concentrations during the 2015 pulse relative to pre- and post-pulse periods (Table 3.1). Relative to the pre- and post-periods there was a greater than 5-fold increase in total pesticide concentrations during the period between May 8 through May 22. Atrazine, acetochlor and metolachlor were consistently the most abundant herbicides measured in POCIS before, during and after the agrichemical pulse. Other pesticides analyzed but not detected in POCIS samplers included alachlor, butylate, chlorthalonil, cyanazine, deisopropylatrazine (DIA), s-ethyl-dipropylthiocarbamate (EPTC), norflurazon, pendamethalin, permethrin, prometon, propachlor, simazine, telfluthrin, trifluralin.

Sediment characteristics and chemistry

Physical analysis of the suspended sediment deposited in the larval mesocosm showed greater accumulation of sediment following high flow events with a relatively

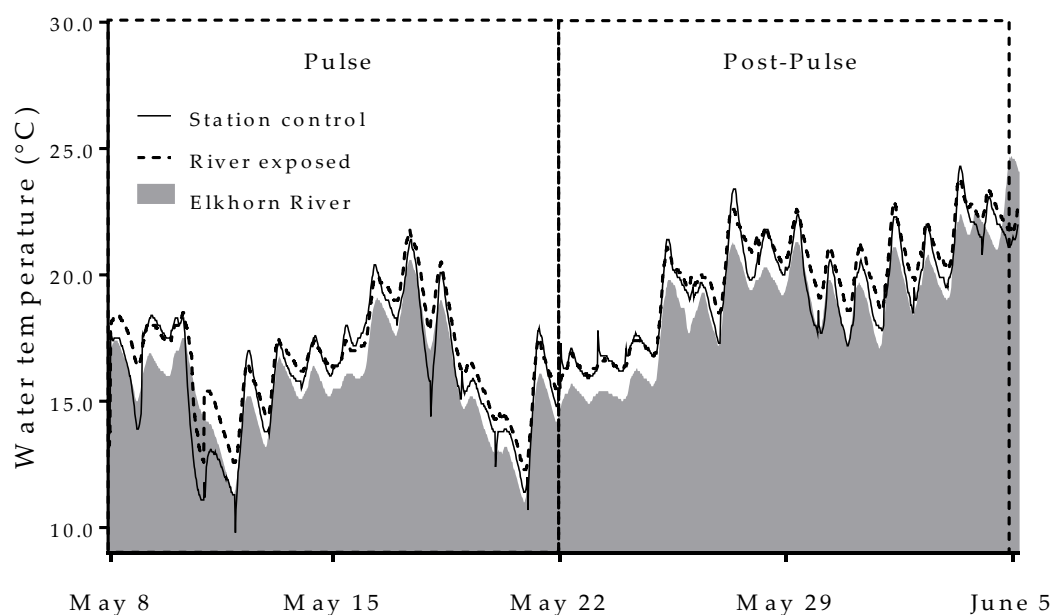


Figure 3. 2 Water Temperature for 2015 Field Exposure

Oscillation in water temperature over the exposure period. Water temperature of the Elkhorn River (solid grey) based on data collected from the USGS Elkhorn River (06800500) gauging Station at Waterloo, Nebraska, USA. Temperature for station control (solid line) and river exposed (dotted line) mesocosms were collected at 30 min intervals using HOBO® data loggers (Bourne, MA).

Table 3. 1 Physicochemical Parameters of Water in the 2015 Exposure

. Water quality and chemical analysis of river water flowing into the larval mesocosm during the exposure period. Water quality parameters presented as the mean (\pm SD). Nanograms (ng) of pesticides in extracts from polar organic chemical integrative samplers (POCIS) deployed across the 2015 agrichemical pulse. Analytical detection limits were determined at <5.0 ng in POCIS.

	April 21- May 5 (Pre- Pulse) ^{a,b}	May 8- May 22 (Pulse)	May 22- June 5 (Post- Pulse)	Fold Change (Pre-pulse to Pulse)	Fold Change (Pulse to Post-pulse)
Temperature (°C)	-	16.70 (2.05)	20.00 (2.08)	-	0.8
Dissolved O ₂ (mg·L ⁻¹)	-	8.42 (1.37)	5.75 (0.73)	-	1.5
pH	-	8.23 (0.16)	8.32 (0.09)	-	1.0
Conductivity (μ S·cm ⁻¹)	-	530.0 (78.0)	584.0 (24.0)	-	0.9
Suspended Solids (mg·L ⁻¹)	-	5,426.7 (3,376.2)	4,460.7 (4,178.5)	-	1.2
Acetochlor	244.2	1775.1	134.6	7.3	13.2
Atrazine	229.4	1517.4	339.9	6.6	4.5
Dimethenamid	75.4	136.1	20.9	1.8	6.5
Metolachlor	197.9	709.7	133.9	3.6	5.3
Deethylatrazine (DEA)	20.2	34.5	24.5	1.7	1.4
Propazine	3.6	23.2	4.3	6.4	5.4
Simazine	10.0	5.3	6.5	0.5	0.8
Metribuzin	< 5.0	14.3	< 5.0	-	

^a '-' Indicates not measured

^b < 5.0 - Below estimated
detection limits.

consistent composition over the 28-d period (Table 3.2). Two to four times more sediment was accumulated in the river water mesocosm in the first two weeks relative to the second half of the exposure. As would be expected, during weeks with reduced river discharge there was a reduction in sand fraction and an increase in the silt fraction. The percentage of clay and TOC remained stable throughout the entire exposure.

The herbicides atrazine, acetochlor, deethylatrazine and metolachlor were detected in sediment samples collected over the course of the 28-d exposure (Table 3.2). The highest concentrations of sediment-associated herbicides occurred during the third sampling period (S3) following the pulse period. This increase in sediment associated agrichemicals was discordant with the peak in agrichemicals observed in the water chemistry during the two-week pulse period (Table 3.1). Other pesticides analyzed but not detected in sediment samples included alachlor, butylate, chlorthalonil, cyanazine, DIA, dimethenamid, EPTC, metribuzin, pendamethalin, permethrin, prometon, propachlor, propazine, simazine, telfluthrin, trifluralin.

Biological effects

Immediately following exposure to the Elkhorn River agrichemical pulse there were differences in growth between station control and river exposed larvae at 19 dph that were reversed by 33 dph (Figure 3.3). At the end of Exposure 1 (19 dph), river exposed larvae had a reduced body mass (t-test, $df=70$; $p<0.001$) and condition factor (t-test, $df=69$; $p<0.001$) relative to station controls. No significant difference in larval body

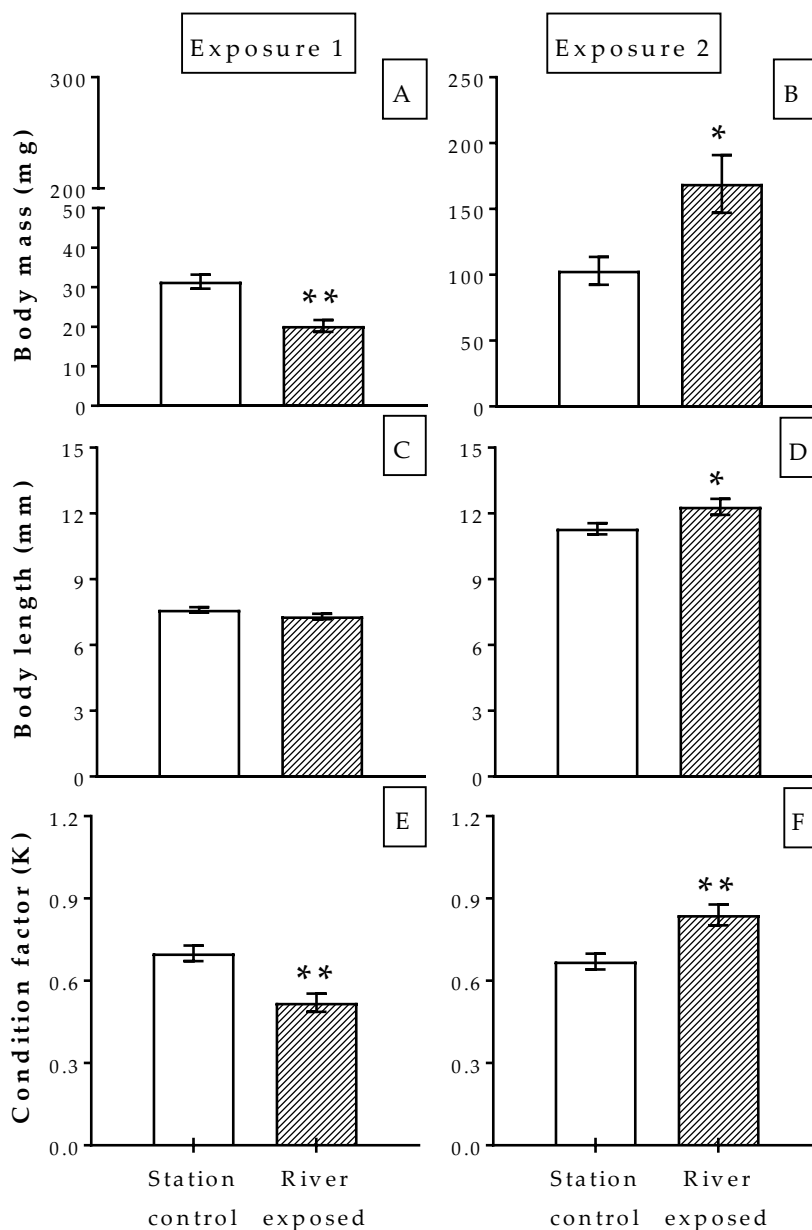


Figure 3.3 Morphometric Analysis of 2015 Minnow Larvae

Morphometric results for station control (empty bar) and river exposed (filled bar) fathead minnow larvae at the end of Exposures 1 and 2 (see Figure 1 for description). Body mass (A), fork length (C) and condition factor (E) for station control and river exposed larvae following Exposure 1 (5-19 dph; n=35-36) and body mass (B), fork length (D) and condition factor (F) following Exposure 2 (5-33 dph; n=20). Values presented as mean (\pm SEM) with significant differences at $\alpha=0.05$ and $\alpha=0.01$ denoted by single and double asterisks, respectively

Table 3. 2 Physicochemical Parameters of the Sediment in the 2015 Exposure

Physical and pesticide analysis of sediments obtained from within the larval mesocosm over the course of the 2015 field season.

Collection Date	Pulse		Post-Pulse	
	May 13 (S1)	May 20 (S2)	May 27 (S3)	June 3 (S4)
Mass (g)	2,551.27	1,140.95	631.57	403.70
% Sand	10	3	3	8
Texture ^a % Silt	67	73	71	72
% Clay	23	24	26	20
Total Organic Carbon (%)	0.18	0.21	0.23	0.15
Pesticides (ng·g ⁻¹) ^b				
Acetochlor	< 0.3	< 0.3	3.92	< 0.3
Atrazine	7.32	7.50	8.74	3.12
Deethylatrazine (DEA)	< 1.4	< 1.4	2.32	< 1.4
Metolachlor	1.49	2.94	3.47	1.09

^a Using standard methods for sieve and hydrometer analysis.

^b "<" Below method detection limits (ng·g⁻¹) See Supplemental Table 1.

length was detected at 19 dph. Conversely, by the end of Exposure 2 (33 dph) the river exposed larvae were significantly larger than station controls in terms of body mass (t-test, Welch's correction, $df=27.52$; $p=0.011$), length (t-test, $df=38$; $p=0.038$) and condition factor (t-test, $df=38$; $p=0.001$). By the end of the 28-d exposure there was 25% and 8% mortality of station control and river exposed larvae, respectively.

Compared to station controls, river exposed fathead minnow larvae showed significant changes in the whole-body expression of endocrine responsive genes following the pulse (Exposure 1) and post-pulse (Exposure 2) exposures (Figure 3.4). At the end of Exposure 1 (5-19 dph), larvae had an upregulation of *igf1* (t-test, Welch's correction $df=22$; $p=0.013$) and a downregulation of *ar* (t-test, $df=21$; $p=0.044$). No difference was detected in the expression of *cyp19a*, *dmrt1*, *era* and *hsd17b* at the end of Exposure 1 (Table 3.3).

At the end of Exposure 2 (5-33 dph), river exposed larvae maintained the upregulation of *igf1* relative to controls at 33 dph (Figure 3.4; t-test, $df=34$; $p<0.001$). However, by the end of Exposure 2 the river exposed fathead minnow larvae experienced a significant upregulation of *ar* expression relative to station controls (t-test, $df=34$; $p<0.0076$). No differences were detected in the expression of the steroidogenic genes *hsd17b* and *cyp19a*, as well as genes *dmrt1* and *era* (Table 3.3).

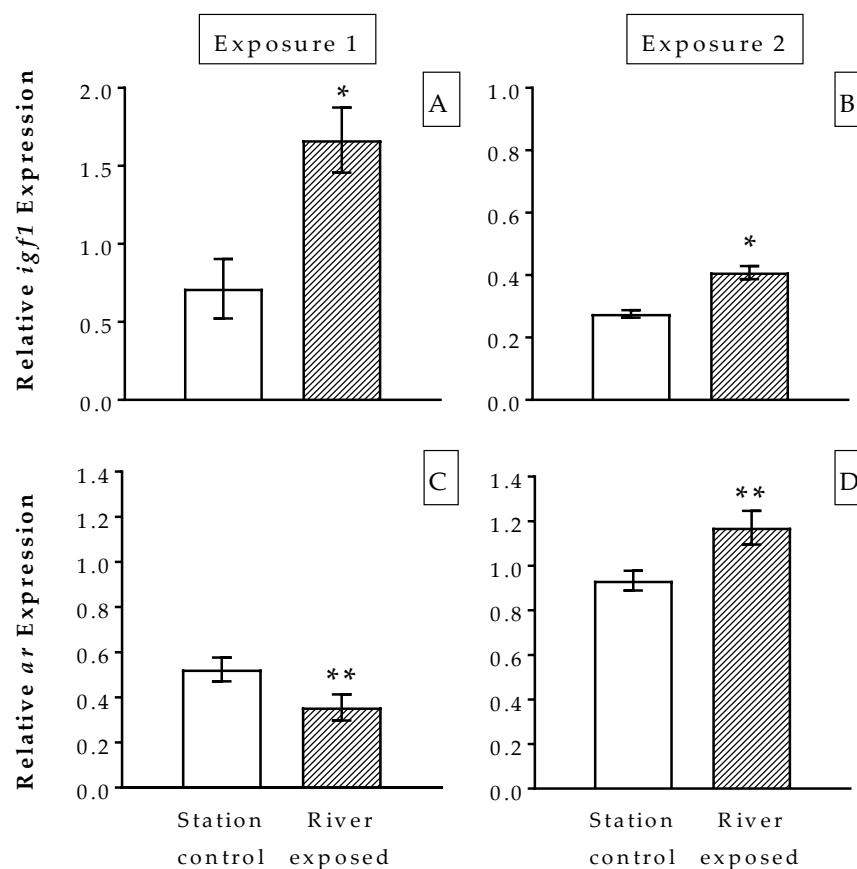


Figure 3. 4 Gene Expression Analysis for 2015 Minnow Larvae

Relative RNA expression in station control (empty bar) and river exposed (filled bar) fathead minnow larvae at the end of Exposures 1 and 2 (see Figure 1 for description). Mean expression values (\pm SEM) for insulin-like growth factor 1 (IGF1) following Exposure 1 (A) and Exposure 2 (B). Mean expression values (\pm SEM) for androgen receptor (AR) following Exposure 1 (C) and Exposure 2 (D). Sample size of 10-12 per treatment group in Exposure 1 and $n=16-20$ in Exposure 2. Significant differences at $\alpha=0.05$ and $\alpha=0.01$ denoted by single and double asterisks, respectively

Table 3. 3 Summary Table of 2015 Exposure Study

Summary of apical and molecular endpoints observed in river exposed larvae relative to their station control counterparts. Significant increases (↑) and decreases (↓) represented by arrows and no significant differences denoted by dash (-) ($\alpha=0.05$).

	Exposure 1 (Pulse, 19 dph)	Exposure 2 (Post-Pulse, 33 dph)
Apical Endpoints		
Body mass	↓	↑
Body length	-	↑
Condition factor (K)	↓	↑
Molecular Endpoints		
Androgen receptor (<i>ar</i>)	↓	↑
Insulin-like growth factor 1 (<i>igf1</i>)	↑	↑
Estrogen receptor α (<i>era</i>)	-	-
Doublesex mab-3 related transcription factor 1 (<i>dmrt1</i>)	-	-
Gonadal aromatase (<i>cyp19a</i>)	-	-
17 β -Hydroxysteroid dehydrogenase (<i>hsd17b</i>)	-	-
"dph" – days post-hatch		

Discussion

The objective of this study was to characterize the developmental plasticity of fathead minnow larvae in a natural environment subject to a seasonal episodic perturbation in the form of a complex mixture of agricultural stressors. It was hypothesized that one of the short-term agricultural pulses would elicit a down regulation in endocrine gene expression and growth, and that post- pulse the larvae would compensate. Larvae exposed to a 14-d pulse showed suppressed endocrine gene expression and growth. Their response to the pulse induced suppression was an over compensation in both growth and endocrine function.

Agrichemical runoff in the Elkhorn River

Although the sampling time periods for the POCIS were different from that for the sediment (Figure 3.1), both illustrate short term changes in the chemical environment that larvae were exposed to. Changes in aqueous pesticide concentrations observed in this study are consistent with previously published differences in agrichemical concentrations during a discharge event relative to post-discharge. Knight et al. (2013) used POCIS samplers to compare agrichemical concentrations during and after a discharge event. The discharge event led to 1.6 to 28-fold increases in herbicide concentrations relative to post-discharge. Similarly, the present study observed increases (up to 13-fold) in atrazine, acetochlor and metolachlor (Table 3.2). These three herbicides represent a signature combination of pollutants found in runoff from corn and soybean

production (Lerch et al. 2011a; Lerch et al. 2011b; Ali and Kolok 2015; Zhang et al. 2015; Fairbairn et al. 2016). The nearly identical concentration of herbicides before and after the discharge event highlights the ephemeral occurrence of waterborne agrichemicals in this watershed.

As seen in previous studies (Ali and Kolok 2015; Zhang et al. 2015), increases in river discharge were accompanied with increases in waterborne agrichemicals as well as the mobilization of sediments and other suspended solids carrying their own pesticide burden. Chemical analysis of accumulated suspended sediments revealed a mismatch between the peaks of pesticides in water and sediment over the 28-d sampling period (Table 3.2; Table 3.3). Specifically, the highest concentrations of waterborne pesticides coincided with the major discharge event whereas sediment-associated pesticide concentrations were the greatest during a lesser spike in river discharge during the post-pulse period, S3 (Figure 3.1; Table 3.3). One plausible explanation for observed differences in aqueous and sediment-associated pesticide concentrations is that a fraction of dissolved pesticides entering the Elkhorn River during the pulse period partitioned into the sediment, which was later mobilized as suspended solids during the lesser discharge event (S3). The role of sediments in the kinetics of agrichemicals in the Elkhorn River has been previously documented (Kolok et al. 2014; Zhang et al. 2015).

The aquatic environments of the pulse and post-pulse periods were distinct from one another not only in their pesticide profiles but in the fluctuations of several physicochemical parameters that can affect aquatic biota. This was evident by measured

differences in conductance, dissolved oxygen levels, and accumulated mass of suspended solids between the pulse and post-pulse periods (Table 3.1; Table 3.2). All of these abiotic factors varied relative to river discharge (Kolok et al. 2014; Kjelland et al. 2015). Biological responses of fish living in this environment would be expected to be a function of these changing physicochemical parameters, as well as diurnal and seasonal (vernal) changes in temperature (Figure 3.2) and photoperiod, (Clark et al. 2005; Blanco-Vives et al. 2011; Ali and Kolok 2015) regardless of the presence of any agrichemicals in the water. This underscores the importance of *in situ* studies for understanding the impacts of natural, composite exposures, as these are events that are not readily simulated under laboratory conditions.

Biological response of larvae to an episodic exposure

The present study documented an interesting dynamic between the expression of AR and IGF1 in fathead minnow larvae following exposure to a seasonal discharge event. Specifically, this is of interest due to their endocrine interactions as well as their responsiveness to environmental stimuli, including endocrine disrupting agrichemicals.

At the molecular level, peptide and steroid hormones facilitate the integration of environmental stimuli with the regulatory mechanisms of multiple physiological systems (Bradshaw 2007; Pittman et al 2013). The peptide hormone, igf1, has endocrine, paracrine and autocrine activity which is readily upregulated or downregulated in response to environmental factors including changes in salinity, nutritional status,

temperature and photoperiod (reviewed by Reinecke 2010). As environmental conditions become optimal for survival and development of an organism, growth will occur. Warmer water temperatures stimulate growth in rainbow trout (*Oncorhynchus mykiss*) through the increased release of growth hormone and subsequent stimulation of igf1 (Gabillard et al. 2003). The strong association between igf1 and growth in bony fish has led to the application of *igf1* mRNA as a molecular marker of growth under a variety of environmental manipulations (Vera Cruz et al. 2006; Montserrat et al. 2007; Vera Cruz et al. 2009; Reinecke 2010; Picha et al. 2014). However, it has been demonstrated that nuclear receptors also modulate the expression of *igf1* following activation by steroid hormones (Riley et al. 2004; Johns et al. 2009; Norbeck and Sheridan 2011; Cleveland and Weber 2015). Immature coho salmon (*Oncorhynchus kisutch*) injected with 11-ketotestosterone or testosterone had a significant increase of circulating igf1 protein one to two weeks following treatment (Larsen et al. 2004). More recently, analysis of hepatic gene expression in female rainbow trout injected with either 17 β -estradiol or dihydrotestosterone demonstrated that androgens stimulate igf1 expression whereas estrogens suppressed *igf1* expression (Cleveland and Weber 2015).

The link between igf1 regulation and sex steroids in fish leaves their early life growth and development susceptible to endocrine disruption by steroidogenic contaminants, such as agrichemicals; however, effects may be confounded by environmental conditions. Sustained *in situ* exposure of juvenile fathead minnows to agricultural ditch water containing a mixture of androgens, estrogens and pesticides lead to increased body size along with male-biased sex ratios at the end of a 6-week

study (Leet et al. 2012). During a subsequent 45-day laboratory study, an increase in the body mass and length of fathead minnows when exposed to a simulated mixture of agrichemicals was observed, but changes in steroid responsive genes at 20 dph or final sex ratio (Leet et al. 2015) was not detected. Leet et al. (2015) speculated that the disparity between outcomes from the *in situ* (Leet et al. 2012) and laboratory studies (Leet et al. 2015) was due to the absence of environmental factors such as spikes in temperature or sediment interactions which may contribute to the overall response of larval fish towards endocrine disrupting chemicals. In these studies, endpoint analysis demonstrated an effect of agrichemicals on growth and development, however they overlook the developmental response that occurs with the exposure.

The present study documents compensatory growth in fathead minnow larvae following exposure to a seasonal discharge event. Immediately after the major discharge event (Exposure 1; Figure 3.1), river exposed larvae displayed suppressed growth and endocrine gene expression as determined by body mass and androgen receptor expression, respectively (Table 3.3; Figures 3.3 and 3.4). This suppression of growth and endocrine function was reversed at the end of a depuration period following the main discharge event which allowed river exposed larvae to achieve greater growth and androgenic gene expression (i.e. masculinization), relative to station controls. Curiously, river exposed larvae maintained elevated igf1 expression throughout the study which was discordant with the observed compensation in other biological endpoints. An explanation for this discrepancy is that induction of compensatory growth by the growth hormone-igf1 axis is not directly mediated by igf1 (Beckman 2011), rather the

induction of compensatory growth is controlled by multiple endocrine and paracrine mechanisms (Won and Borski 2013). To our knowledge, this is the first study to characterize compensation of larval fish following an *in situ* episodic exposure to seasonally occurring agrichemical stressor.

Given that molecular defeminization has been consistently documented in adult fathead minnows following exposure to an agrichemical pulse (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015), our initial question was whether we would see a similar anti-estrogenic profile in larval fathead minnows. Both Knight et al. (2013) and Ali and Kolok (2015) found that the pulse-associated defeminization of adult fish was absent during post-pulse periods with reduced concentrations of agrichemicals, similar to the post-pulse concentrations seen in this study. In a subsequent study on the Elkhorn River, Zhang et al. (2015) found that agrichemical-laden sediments carried by pulse events were responsible for reductions in hepatic estrogen- and androgen-receptor expression in adult fish. The downregulation of androgen receptor expression in adult fish (Zhang et al. 2015) is consistent with the response observed in the present study immediately following the pulse. It is likely that the absence of defeminization of minnow larvae in the present study is due to either environmental or biological factors. While Zhang et al. (2015) confirmed that sediment was a major source of agrichemical exposure for adult fish, this may not hold true for larvae whose interactions with sediment and water may differ from adult fish. A biological explanation for the absence of defeminization is that the expression profile of estrogen receptors in fathead minnow, namely receptor subtypes α , β and γ , has been shown to vary over the course of

ontogeny which would determine what type of response larvae and juvenile fish might have towards an anti-estrogenic mixture (Filby and Tyler 2007; Johns et al. 2011; Leet et al. 2013). Therefore, while subtle differences were observed between the responses of larvae from the present study and those observed in adult fish from previous studies there is evidence for a consistent response of fish towards agrichemical pulses. Future studies are needed to investigate the impacts of similar exposures on multiple levels of endocrine activity and the mechanisms by which compensatory responses occur.

CHAPTER 4: RESPONSE AND RECOVERY OF FATHEAD MINNOWS (*PIMEPHALES
PROMELAS*) FOLLOWING EARLY LIFE EXPOSURE TO WATER AND SEDIMENT
FOUND WITHIN AGRICULTURAL RUNOFF FROM THE ELKHORN RIVER,
NEBRASKA, USA.

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Introduction

Agricultural runoff contains a mixture of chemical contaminants that partition between the aqueous phase and the suspended sediment phase. The aqueous phase of agricultural runoff is generated by snowmelt and rainfall events that coincide with the seasonal application of fertilizers (Gall et al. 2015; Yang et al. 2016), pesticides (Schulz 2004; Lerch 2011a,b) and manure containing veterinary pharmaceuticals (Bernot et al. 2013; Joy et al. 2013; Jaimes-Correa et al. 2015) and natural steroids (Gall et al. 2011; Mansell et al. 2011). Polar hydrophilic contaminants are readily dissolved into the aqueous matrix, facilitating for their downstream transport within receiving surface waters. Agrichemical contaminants from the aqueous phase can also partition into the suspended sediment phase depending on several physicochemical properties (Hallare et al. 2011; Fairbairn et al. 2015; Sangster et al. 2015; Zhang et al. 2015; Zhang et al. 2016; Sangster et al. 2016). Together, the water-sediment combination found in agricultural runoff presents two major sources for exposure to agrichemical contaminants in aquatic environments.

In the last decade, several studies have demonstrated that fish exposed to agricultural runoff experience endocrine disrupting effects from the macromolecular to organismal level (Orlando et al. 2004; Kolok et al. 2007; Sellin et al. 2010; Sellin Jeffries et al. 2011a,b; Sellin Jeffries et al. 2012; Knight et al. 2013; Blazer et al. 2014; Cavallin et al. 2014; Skelton et al. 2014; Ali and Kolok 2015; Zhang et al. 2015; Ali et al. 2016; Ali et al. 2017). Wild populations of smallmouth bass (*Micropterus dolomieu*) from Pennsylvania

presented an increased incidence of testicular oocytes and elevated vitellogenin (vtg) that was strongly correlated to agricultural land use and the presence of agrichemicals such as steroid hormones, triazine and acetanilide herbicides in both water and sediments (Blazer et al. 2014). Mature female fathead minnows (*Pimephales promelas*) exposed to agrichemical-laden sediments under laboratory and in situ conditions experienced reductions in hepatic expression of the estrogen-responsive genes *vtg* and estrogen receptor α (*era*) (Sellin Jeffries et al. 2011a, 2011b), but had no response to field collected water alone. This anti-estrogenic effect in adult minnows is the most pronounced during spring runoff events when there are elevated aqueous concentrations of agricultural contaminants (Knight et al. 2013; Ali and Kolok 2015) and mobilization of agrichemical-laden sediments (Zhang et al. 2015). Collectively, these studies demonstrate that both the aqueous and sediment matrices play important roles mediating exposure to agrichemical contaminants.

Sediment may be particularly important to larval fish because of their relative sensitivity to stressors, large surface area to body volume ratio and life history traits that involve settling and foraging on sediment beds (Hallare et al. 2011). Fathead minnow larvae were protected from the lethal effects of formononetin and genistein, phytoestrogens detected in pasture runoff, by their sorption to sediment (Kelly et al. 2015) whereas exposure to these same isoflavones without sediment reduced survival following 21-d exposure (Rearick et al. 2014). With respect to agricultural runoff, Leet et al. (2012) found that six-week exposure of minnow larvae (0-40 days post-hatch (dph)) to agricultural ditchwater, containing pesticides, fertilizers, estrogens and androgens,

resulted in enhanced growth and male-biased sex ratios; however the impact of the likely contaminated suspended sediments was not characterized. Conversely, Ali et al. (2017) detected anti-androgenic changes in androgen receptor (AR) mRNA expression in fathead minnow larvae (19 dph) following in situ exposure to a spring agricultural runoff event comprised of agrichemicals, suspended sediments and oscillating physicochemical parameters. Although these field studies (Leet et al. 2012; Ali et al. 2017) and others demonstrated that agricultural contaminants elicit endocrine responses from larval fish, the specific contributions of the water and sediment matrices have yet to be adequately characterized.

As water and sediment are major constituents of a fish's early life environment, it is important to consider how contamination of these matrices can affect larval growth, survival and subsequent developmental outcomes. In the present study, we investigated how early-life exposure to water and suspended sediments found in agricultural runoff affects larval-stage biological responses and adult outcomes in a small fish model, the fathead minnow. Specifically, the two objectives of this experiment were to determine: (1) if exposure to agricultural runoff water, sediment or the water-sediment combination was responsible for the changes in endocrine-response gene expression and development of fathead minnow larvae and (2) whether early-life exposure to water, sediment or the water-sediment combination would produce adverse effects on adult health and reproduction. To address our first objective, we exposed fathead minnow larvae during the first month post-hatch to water and sediment collected from the Elkhorn River, then measured changes in endocrine-responsive gene expression and

morphometric endpoints. Water and sediment exposed larvae were then allowed to depurate in uncontaminated conditions until reaching sexually maturity, at which time adult health outcomes were assessed. This best-case scenario for recovery was selected to isolate the effect of early life exposure to the water and sediment matrices from the effects of chronic life-long exposure to background contamination by other sources of pollution.

Materials and Methods

Fish Production and Maintenance

Fathead minnow larvae (*Pimephales promelas*) used for this experiment were obtained from the Aquatic Toxicology Laboratory (ATL) at the University of Nebraska at Omaha, Omaha, NE. All procedures were conducted in compliance with protocols approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (Protocol #98-075-11). Outside of the 30-d larval exposure described below, all fish were maintained in carbon-filtered dechlorinated (filtered) tap water at 24.4 ± 1.2 °C (mean \pm SD) with static 1/3 renewal of water once daily. All fish older than 30-days post-hatch (dph) were fed Tetramin Flake Food (Melle, Germany), whereas larvae (0-30 dph) were fed daily with < 24 h old brine shrimp (*Artemia nauplii*) (INVE Aquaculture, UT, USA). Beginning on April 20, 2016, breeding triads of adult fathead minnows were established consisting of one male and two females. Fish were housed in 30 L aquaria, divided into two compartments by a plastic, porous divider with two

triads in each aquarium. Breeding triads were provided with a 12-cm half of 8 cm diameter PVC tubing as a breeding tile on which eggs were laid.

After the triads began to breed, the tiles with eggs were removed daily from the aquaria and transferred to 1 L aerated beakers. Unfertilized and fungus infected eggs were removed daily, and surviving embryos were all hatched by 5-days post fertilization. Hatched larvae were transferred to 1 L beakers at a density of 100 larvae per liter and transferred to their respective exposure aquaria described below (see Larval Exposure).

Experimental Design

This experiment utilized a 2 x 2 factorial design to test the effect of exposure to two water treatments, two sediment treatments and the interaction thereof on the biological responses of minnow larvae, and the subsequent effects of these early-life treatments as adults. Water treatments were either “filtered” dechlorinated tap water or “river water” collected from the Elkhorn River during agricultural runoff as described below (see Exposure Set Up). Sediment treatments consisted of either the absence or presence of suspended sediments that were collected from the Elkhorn River during an agricultural runoff event. The cross of the water and sediment treatments produced four treatment groups for the larval exposure that were: filtered water (FW), filtered water with sediment (FWS), river water (RW) and river water with sediment (RWS). Filtered water consisted of dechlorinated, carbon filtered Omaha tap water. To allow for

assessment of survival and adult sex ratio, each of these treatment groups consisted of four technical replicates of each treated aquaria with 100 fathead minnow larvae per replicate aquaria at the start of the exposure for a total of 400 larvae per treatment group.

The early-life exposure was conducted for 30 d between the ages of 0-30 dph. This early-life exposure window was selected since fathead minnows undergo sexual differentiation between 0-30 dph, which presents a susceptible period of sexual differentiation for fathead minnows (Van Aerle et al. 2002; Van Aerle et al. 2004; Leet et al. 2013). Previous studies by our lab have shown that a 30-d exposure is representative for a typical runoff season in the Elkhorn River (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015; Ali et al. 2016; Ali et al. 2017). This allowed the experiment to evaluate a natural runoff scenario for larvae, whereas the subsequent depuration in filtered water allowed for optimal recovery conditions leading into adulthood.

Exposure Microcosms

The water and sediments used in this experiment were collected from the Elkhorn River Research Station (ERRS) located on the Elkhorn River approximately 10 km upstream from the confluence of the Elkhorn and Platte Rivers, Nebraska, USA. This open-air facility has been used for multiple field studies on agricultural runoff and is capable of continuously accumulating suspended sediments from the Elkhorn River in stainless steel chambers (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015; Ali et al. 2017). The schedule for the collection of water and sediment from the ERRS, and

periods from which sediments were accumulated then collected are detailed in Figure 4.1A.

The onset of agricultural runoff was determined by using atrazine test strips to detect the presence of atrazine above $3 \mu\text{g}\cdot\text{L}^{-1}$ which has been shown to serve as a source-specific indicator of seasonal agricultural runoff within Nebraska surface waters (Knight et al. 2013; Ali and Kolok 2015; Ali et al. 2017). The occurrence of two positive atrazine tests in combination with regional precipitation events signaled the onset of spring agricultural runoff and start of the experiment's water and sediment collection for the larval exposure.

Water was collected daily from the ERRS and transported to the ATL, located at the University of Nebraska at Omaha, using 18.9 L bisphenol A-free, opaque, food grade plastic buckets. Suspended sediments were allowed to accumulate within the stainless-steel chambers at the ERRS over a 5-d period then transferred to the ATL for addition to the FWS and RWS aquaria. This allowed for exposure to suspended sediments that were mobilized within agricultural runoff during periods of elevated river discharge which have been shown to elicit biological effects from adult fish in the Elkhorn River (Zhang et al. 2015). The total time in transit from collection at the ERRS until transfer into the aquaria in the ATL was between 2-3 hours, where water and sediments were maintained at ambient temperature while in transit (inside an airconditioned vehicle) and room temperature while in the ATL.

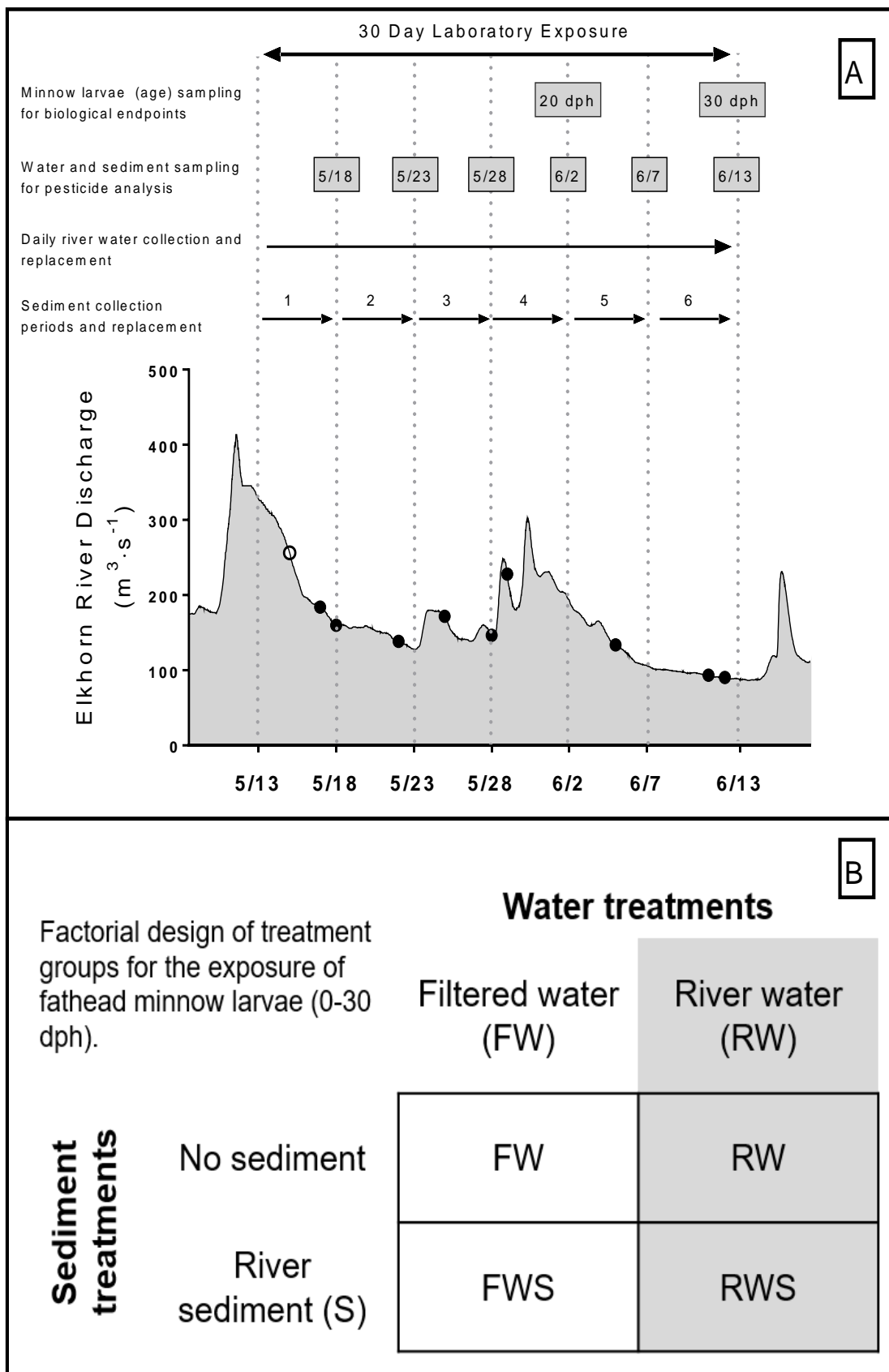


Figure 4. 1 Experimental Design for 2016 Exposure

Environmental sampling (A) and factorial design of treatment groups (B) for the 0-30 d larval exposure. Environmental sampling and matrix collection for 30-d exposure of fathead minnow larvae in the Aquatic Toxicology Laboratory at the University of Nebraska at Omaha (top) relative to temporal changes in Elkhorn River discharge (bottom). Sampling days for biological endpoints and chemical analysis are shown in grey boxes. Sediment collection is shown with arrows indicating the collection period (line) and date the sediment was transferred to the laboratory exposures (end arrow). Temporal changes in discharge are based on data collected from the USGS Elkhorn River (06800500) gaging station at Waterloo, Nebraska, USA. Detection of the runoff period was conducted using atrazine test strips (see Methods). Positive atrazine test strip results are shown as solid circles, and negative test results are represented as open circles. Figure 4.1B depicts the four treatment groups for the larval exposure component with 4 replicate aquaria per treatment group, and 100 larval per replicate aquaria.

Each treatment replicate was established in 45 L aquaria maintained under a 16:8 h light:dark cycle and supplied with an air-stone and a 100 W heater. The FW and RW treatment groups were only supplied with their respective water types which had 15 L (1/3 total volume) exchanged daily with water from their respective sources. The FWS and RWS groups followed the same procedure as the FW and RW groups but started the exposure with the addition of 6 L of whole wet sediment previously collected from the ERRS. For the remainder of the exposure, sediments within each FWS and RWS aquaria were exchanged at a rate of 1 L of whole wet sediment every 5 d with sediments collected from the ERRS.

Water and Sediment Sampling

Water quality parameters including temperature, pH, dissolved oxygen, total dissolved solids and conductivity were measured daily within each replicate aquarium using a YSI ProPlus® Meter (YSI, OH, USA). Alkalinity was determined by titration using a Hach titration kit per manufacturer's protocol. A total of six composite water samples (Figure 4.1) were collected from the FWS, RW and RWS treatment groups using 1 L amber glass bottles and stored at -20 °C for chemical analysis (see below) by the Water Sciences Laboratory (WSL), University of Nebraska-Lincoln.

Composite sediment samples for physical and chemical analysis were collected from the FWS and RWS aquaria with a total of six samples over the course of the 30-d exposure (Figure 4.1). Samples were collected between 5-8 hours after sediment

replacement to integrate the prior sediment profile with the contribution of the added sediments. The composite samples were collected using glass jars and frozen at -20 °C for pesticide analysis by WSL (described below). The remaining sediment was dried at 100°C until a constant mass was reached (12-24 h). Dry sediment samples were analyzed using standard methods to determine texture (Gee and Or 2002) and total organic carbon (Islam and Weil 1998).

Chemical Analysis

A suite of pesticides was selected for quantification given regional land use and the seasonal detection of their presence within the Elkhorn River watershed using previously described methods (Ali and Kolok 2015; Zhang et al. 2015; Ali et al. 2017). Water samples were extracted by solid phase extraction (SPE) for dissolved pesticide analysis (Cassada et al. 1994). Sediment samples were extracted using MASE techniques with a MARS XPress (CEM Corporation, Matthews, NC) microwave system following previously described methods (Cassada et al. 1994; Smalling et al. 2008). Water and sediment extracts were analyzed by gas chromatography mass spectrometry (GC/MS) analysis. Detailed methods for extraction and GC/MC analysis are detailed in the Supplemental Information along with method detection limits (Supplemental Table 3).

Larval Exposure

Fathead minnow larvae were subject to a 30-d exposure from 0 to 30 dph, corresponding to May 14 through June 13. Due to the small size of the minnow larvae, they were maintained in PVC exposure vessels within the exposure aquaria as detailed in the Supplemental Methods (Supplemental Figure 1). For additional details on establishment of the exposure vessels, refer to the Supplemental Material (Larval Exposure Setup). Larvae remained in their same exposure vessel over the course of the 30-d larval exposure. Sediment samples were collected from within a subset of the FWS and RWS exposure vessels on exposure days 10 and 20 for comparison to sediment composition from the outside 45-L aquaria. On days 20 and 30 of the exposure five larvae were randomly selected from each replicate exposure vessel, providing a total of 20 larvae per treatment group, and sacrificed using a lethal dose of MS222 to collect morphometric measurements (see Morphometric Analysis) and whole-body gene expression analysis (see Gene Expression Analysis). Survival was assessed at the end of the exposure (30 dph) by counting the number of larvae remaining in each exposure vessel.

At the end of the 30-d exposure, all larvae were transferred to separate 30 L aquaria containing filtered water and maintained as previously described for adults in Fish Production and Maintenance. These fish were allowed to depurate in filtered water until reaching sexual maturity (165 dph). Upon reaching sexual maturity, minnows were either subjected to a 21-d breeding assay (described below) or sacrificed using a lethal

dose of MS222 to collect morphometric measurements and determine final sex ratio of each treatment replicate.

Gene Expression Analysis

Whole minnow larvae bodies were collected at 20 and 30 dph for reverse transcription quantitative polymerase chain reaction (RT-qPCR). RNA extraction, purification, quality control and cDNA synthesis followed previously described methods for fathead minnow larvae (Ali et al. 2017). All PCR reactions were performed using the CFX Connect Real-Time PCR Detection System using the iTaq Universal SYBR® Green Supermix, 2x concentration, (Bio-Rad, California, USA) per the manufacturer's protocol using 30 ng cDNA template per reaction. Given the role of androgens and estrogens in sexual differentiation, primary genes of interest included androgen receptor (*ar*), estrogen receptor α (*era*), estrogen receptor β (*er β*). Additionally, expression of insulin-like growth factor 1 (*igf1*) and glucocorticoid receptor (*gr*) were quantified to determine if exposure to water or sediments affected expression of genes involved in growth and stress response. All gene expression was normalized using the reference gene ribosomal protein L8 (*rpl8*) (Kolok et al. 2007; Ali et al. 2017). Specific primer sequences (Kolok et al. 2007; Filby and Tyler 2007; Beggel et al. 2011) and PCR reaction efficiencies are provided in Supplemental Table 4.

Morphometric Analysis

Both larval and adult fathead minnows were assessed for body mass (g), length (cm), and condition factor (K) where $K = (\text{body mass (g)} / \text{body length (cm)}^3) \times 100$. Primary sex characteristics of adult fish were assessed through gonadosomatic index (GSI) where $GSI = (\text{gonad mass (g)} / \text{body mass (g)}) \times 100$ and final sex ratio (proportion female) as determined by visual inspection upon dissection. Secondary sex characteristics in both adult male and female minnows included tubercle counts (a marker of masculinization) and the ovipositor length (a female-specific external structure for egg laying) relative to whole body length. Tubercles were counted by visual inspection, whereas ovipositor length was measured using digital calipers from the base of the structure to the tip.

Reproductive Assessment

Upon reaching sexual maturity (165 dph), minnows were segregated into four breeding groups based on their prior exposure: lab water (FW), lab water and sediment (FWS), river water (RW) and river water and sediment (RWS). Three tanks were assigned per breeding group and each breeding group consisted of a triad of fish, 2 females and 1 male, thereby providing a total of 6 breeding triads per breeding group with a total of 24 breeding triads. Triads were maintained in 30-L tanks that contained two triads separated by a plastic divider.

Reproductive assessment of each triad was conducted using a modified version of the 21-d breeding assay (Ankley et al. 2001). After a 7-d acclimation period, spawning events were recorded daily after 11:00 AM. Spawning frequency was calculated as the number of pairs to spawn per day. Spawning substrates with adhered eggs were removed and replaced from each tank and placed in a 1-L beaker containing clean laboratory water maintained in a warm water bath at 24.9 ± 0.9 °C. Due to limited space for maintenance of egg maintenance, fertilization was assessed once per week for each treatment group. Clutch size, the number of eggs on each substrate, was evaluated so that fecundity could be determined. Fecundity was measured as the number of eggs laid per day per number of females. Fertilization was determined weekly by counting the number of eggs with eye spots at 96 h post-spawn. Fertilization success was measured as the percent of eggs fertilized in each clutch.

Statistical Analysis

Data were analyzed using JMP 11 software (SAS, NC, USA). All biological data were compared using two-way ANOVA to test for an effect of water source (filtered water or river water), sediment presence (i.e. absence or presence) or the interaction there of, with sample sizes reported in respective figure captions. Post-hoc analyses were conducted using t-test where an effect of either water or sediment treatment alone was detected, whereas Tukey's test was used where an interaction effect was detected. Data collected from 20 dph and 30 dph larvae were analyzed separately, with no

comparisons between these two age groups. Morphometric and reproductive assessment data, except for fertilization, were compared between treatment groups without violation assumptions for parametric analysis. Relative gene expression data were log transformed to satisfy assumptions of normality and equal variance. Proportional data for final sex ratios (p female), survival and adult fertilization score were arcsine transformed to satisfy parametric assumptions for two-way ANOVA (Sokal and Rohlf 1995). Sample sizes for each analyzed endpoint are provided in Supplemental Tables 6 and 7. Statistical significance was assumed at $p < 0.05$.

Results and Discussion

The two objectives of this experiment were to determine (1) if exposure to agricultural runoff water, sediment or the water-sediment combination was responsible for the changes in endocrine-responsive gene expression and development of fathead minnow larvae and (2) whether early-life exposure to water, sediment or the water-sediment combination would produce adverse effects on adult health outcomes and reproduction. This was accomplished by evaluating the biological responses of fathead minnow larvae that were exposed to water, sediment or the water-sediment combination collected from agricultural runoff for 30 d followed by a depuration period until the fish reached sexual maturity. The outcomes of this study were the observation of agrichemical partitioning between the water and sediment matrices and the detection

of matrix-specific responses in larval fish that were previously only demonstrated in adult fish.

Pesticides in the Water and Sediment Matrices

Row crop production found throughout eastern Nebraska and the Midwestern United States relies on intensive use of pre-emergent herbicides including triazine and acetanilide herbicides (Ryberg and Gilliom 2015). The most regularly detected herbicide was atrazine, followed by acetochlor and metolachlor (Table 4.1), which was expected as this trio serves as a source-specific indicator for spring runoff in agriculturally-dominated watersheds (Lerch et al. 2011b; Hyer et al. 2001; Ali and Kolok 2015; Ryberg and Gilliom 2015; Fairbairn et al. 2016). Zhang et al. (2015) reported that 77-89% of the total dissolved pesticide profile found in the Elkhorn River from early May through June 2014, consisted of atrazine, acetochlor and metolachlor, and their concentrations ranged from 0.66 to 14.8 $\mu\text{g}\cdot\text{L}^{-1}$. The aqueous concentrations of herbicides measured in the present study fall within the estimated annual ranges of atrazine (0.029-149 $\mu\text{g}\cdot\text{L}^{-1}$), acetochlor (<0.001-3.80 $\mu\text{g}\cdot\text{L}^{-1}$) and metolachlor (<0.001-90.1 $\mu\text{g}\cdot\text{L}^{-1}$) (Lerch et al. 2011a, 2011b), although more recent surveys (Ryberg and Gilliom 2015) suggest downward trends in usage and release of these herbicides in certain watersheds. Identification of agricultural runoff, aided by the atrazine test strip in combination with GC-MS analysis, confirmed that the concentrations and compounds identified during the exposure period

Table 4. 1 Pesticide Profile for 2016 Water and Sediment

Summary of the pesticide profile for river water and suspended sediments collected from the 30-d larval exposure. River mean concentrations and % determined from both RW and RWS aqueous samples. Sediment mean concentrations and % determined from both FWS and RWS sediment samples based on wet weight. Values presented as the mean (\pm standard deviation) from samples collect across the 30-d exposure period.

Compound	Class ^a	log K _{ow} ^b	River water				Sediment			
			Mean $\mu\text{g}\cdot\text{L}^{-1}$		Mean %		Mean $\text{ng}\cdot\text{g}^{-1}$		Mean %	
Atrazine	T	2.68	1.99	(1.69)	53.9	(4.9)	7.76	(3.49)	38.9	(5.0)
Acetochlor	A	2.48	0.75	(0.84)	16.4	(7.1)	4.56	(2.52)	22.6	(7.2)
Metolachlor	A	2.90	0.50	(0.47)	14.4	(4.0)	4.12	(1.74)	21.2	(3.4)
DEA	T	1.51	0.22	(0.14)	8.7	(5.3)	0.51	(0.40)	2.5	(1.5)
Dimethenamid	A	2.14	0.12	(0.07)	5.2	(3.9)	0.54	(0.55)	2.4	(1.5)
Propazine	T	2.93	0.02	(0.02)	0.8	(0.3)	0.18	(0.21)	0.8	(0.9)
DIA	T	1.15	0.03	(0.07)	0.4	(0.8)	-		-	
Metribuzin	To	1.70	0.01	(0.01)	0.2	(0.2)	0.24	(0.82)	1.4	(4.7)
Alachlor	A	3.52	0.00	(0.00)	0.1	(0.2)	0.20	(0.68)	0.9	(3.2)
Pendamehalin	A	5.20	-		-		1.59	(0.56)	8.5	(2.5)
Tefluthrin	P	6.40	-		-		0.08	(0.17)	0.4	(0.8)
Trifluralin	D	5.34	-		-		0.10	(0.06)	0.4	(0.3)

- indicates not detected.

^a T= Triazine, To=Triazone, A=Acetanilide, P=Pyrethroid, D=Dinitroaniline

^b K_{ow} values derived from literature reported values (Finizizio et al. 1991; Hansch et al. 1995; Tomlin 2004; Van der Merwe and Riviere 2005; Bedmar et al. 2011; Memic et al. 2011; Westra 2012;).

in the present study were consistent with previous studies of seasonal agricultural runoff at this location.

As in previous studies on runoff in the Elkhorn River (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015; Ali et al. 2017), aqueous pesticide concentrations were positively associated with discharge ranging from 0.1 to 8.3 $\mu\text{g}\cdot\text{L}^{-1}$ (Figures 4.1 and 4.2). The peak in dissolved pesticide concentrations found in the river water treated aquaria, river water (RW) and river water with sediment (RWS), coincided with elevated river discharge that occurred between May 28 through June 2, 2016. Despite supplementation of contaminated sediments in the RWS aquaria, total concentrations of dissolved pesticides in the RW and RWS aquaria were nearly identical, indicating a consistent exposure in terms of aqueous agrichemicals and other water quality parameters (Supplemental Table 5). The addition of sediment to filter water (FWS) contributed a negligible amount of pesticides to the filtered water matrix over the 30-d exposure, likely due to minimal dissociation of relatively more hydrophobic compounds from the supplemented sediments.

Although the sediment did not have an appreciable effect on aqueous pesticide concentrations, river water and filtered water influenced the dynamics of pesticide accumulation in the sediment (Figure 4.2). Pesticide concentrations were regularly measured at or above 9.7 $\text{ng}\cdot\text{g}^{-1}$ in the sediment matrix comprised of silt ($49.2 \pm 10.1\%$), sand ($44.7 \pm 11.1\%$) and clay ($6.1 \pm 2.7\%$), with a lesser contribution from total organic carbon ($0.13 \pm 0.05\%$). While these concentrations of sediment-associated agrichemicals

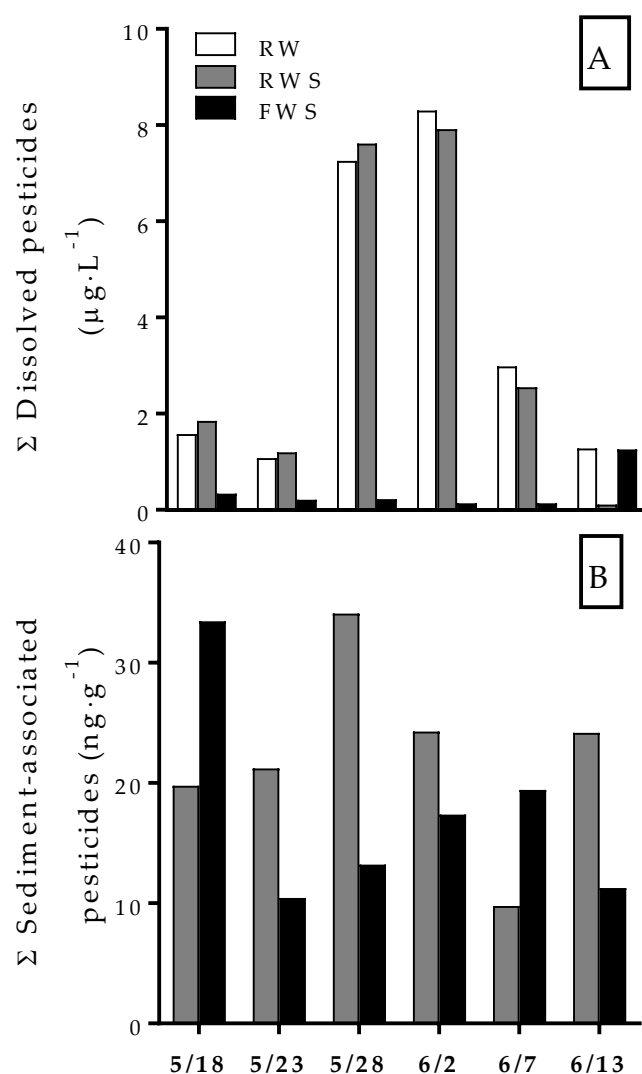


Figure 4. 2 Cumulative Pesticide Concentrations in 2016 Treatment Groups

. Concentrations of pesticides detected by gas chromatography mass (GC/MS) spectrometry in composite water (A) and sediment (B) samples taken from aquaria over the 30-d larval exposure. Bars represent total concentration of all detected pesticides with river water (RW), river water with sediment (RWS) or filtered water with sediment (FWS).

were consistent with those reported two-years prior by Zhang et al. (2015), the concentrations of herbicides such as atrazine and acetochlor were higher than those measured in less agriculturally-intensive region of the Midwest, such as Minnesota (Fairbairn et al. 2015). The sediment pesticide profile contained the same agrichemicals as the aqueous matrix as well as more moderately hydrophobic compounds ($K_{ow} > 5.0$), emphasizing the capacity for sediment to act as a sink for organic compounds. In the aquaria containing both river water and sediments (RWS), peaks in sediment-associated pesticide concentrations closely followed those of the aqueous phase suggesting that pesticides present in the river water partitioned into the sediment. Conversely, sediment concentrations of pesticides in the FWS aquaria were high at the beginning of the exposure but stabilized over time with minimal losses of hydrophilic parent compounds and their metabolites to the filtered water phase, a phenomenon which has been previously reviewed by Kolok et al. (2014).

The high variation of aqueous pesticide concentrations relative to those measured in sediments supports the contention that the profile of agrichemicals in surface water is discordant from that of sediments (Ali et al. 2017; Kolok et al. 2014). This discordant timing of persistent sediment-associated agrichemicals also presents a potential chronic exposure relative to the more episodic concentrations found in the aqueous matrix. Thus, investigation of agricultural runoff requires consideration of the specific exposure matrix as well as temporal variability associated with fate and transport in riverine environments.

Impact of River Water and Sediment on Minnow Larvae

In the present study, the constituent water and sediments found in agricultural runoff elicited matrix specific effects on early life growth and endocrine-responsive gene expression in fathead minnow larvae. These macromolecular effects were either anti-androgenic or anti-estrogenic responses that were associated with the water matrix and sediment matrix, respectively.

The aqueous matrix of the Elkhorn River's spring agricultural runoff elicited the upregulation of androgen receptor expression and increased the average size of fathead minnow larvae. Minnow larvae exposed to the Elkhorn River water alone (RW treatment group) exhibited larger body masses at 20 dph (two-way ANOVA, $F=3.528$, $p=0.0188$; interaction term, $F=3.528$, $p=0.0188$) and 30 dph (two-way ANOVA, $F=6.040$, $p=0.0006$; interaction term, $F=11.937$; $p=0.0007$) relative to filtered water controls (Figure 4.3), with a similar response in body length at both ages (Supplemental Table 6; Supplemental Figure 2). In the river water aquaria supplemented with sediment (RWS treatment group), minnow larvae did not exhibit the same increase in body mass as the RW treated larvae. However, both RW and RWS treated larvae experienced a reduction in body condition factor (K) by 30 dph (two-way ANOVA, $F=7.020$, $p=0.0002$; water source effect, $F=5.883$, $p=0.0164$). Thus, while river water exposed larvae were heavier and longer than their counterparts, they were not an appropriate weight for their size

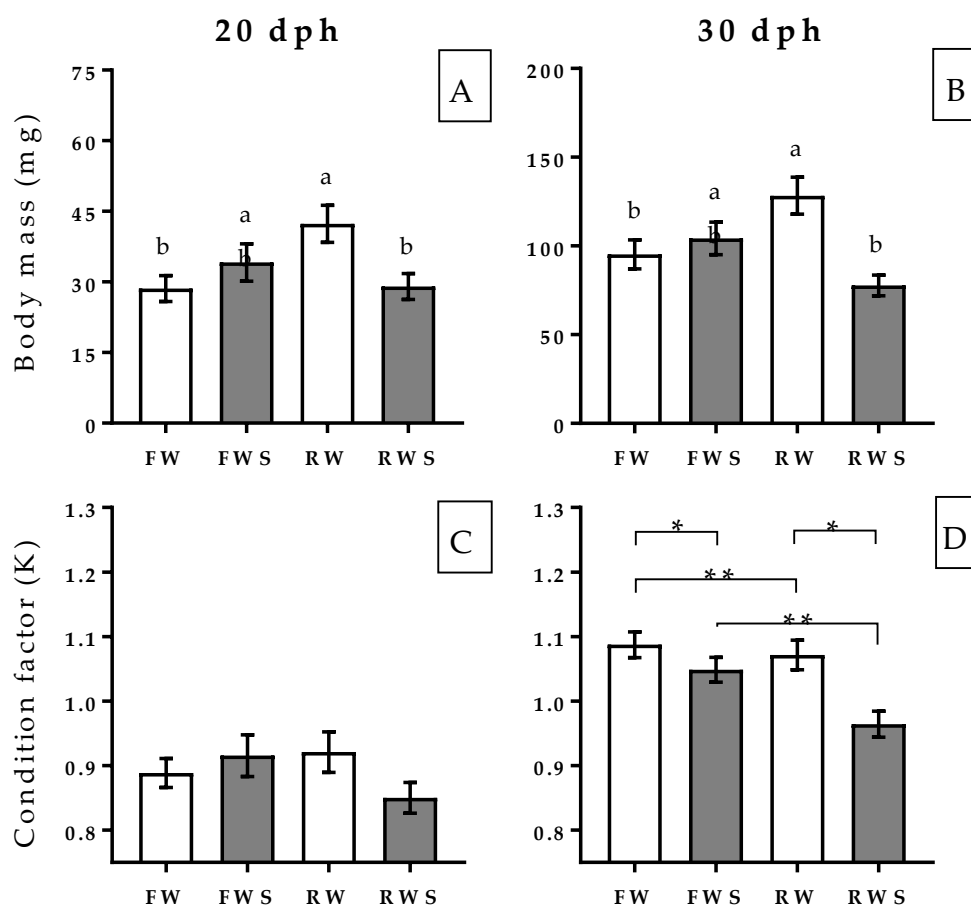


Figure 4.3 Morphometric Analysis of 2016 Minnow Larvae

Morphometric results for 20-dph differences in body mass (A) and condition factor (C) along with 30-dph differences in body mass (B) and condition factor (D). Treatment groups on the x-axis are filtered water (FW), filtered water with sediment (FWS), river water (RW) and river water with sediment (RWS). Bars represent the mean (\pm SEM; $n=20$ per treatment group) of individual larvae from each treatment group. Significant differences detected by Tukey's test for differences due to an interaction effect are denoted by lower case letters, whereas differences detected by t-test are indicated by brackets with single asterisk for water type effect or double asterisks for sediment treatment effects ($\alpha=0.05$).

suggesting less than optimal growth. There was no effect of water or sediment treatment on larval survival by the end of the 30-d exposure (Supplemental Table 6).

At the macromolecular level, treatment with river water (RW and RWS) upregulated androgen receptor (*ar*) mRNA expression at 20 dph (two-way ANOVA, $F=6.985$; $p=0.0010$; water source effect, $F=19.106$, $p=0.0001$) and 30 dph (two-way ANOVA, $F=4.383$, $p=0.0113$; water source effect, $F=12.295$; $p=0.0015$) (Figure 4.4), but no effect on other endocrine responsive genes (Supplemental Table 6). These observations are consistent with a prior field study conducted on the Elkhorn River where fathead minnow larvae (33 dph) experienced similar upregulations in *ar* gene expression and body mass following a 28-d in situ exposure to agricultural runoff (Ali et al. 2017). Similar upregulations of *ar* gene expression in adult and larval fish have been reported following exposure to known androgen antagonists such as methoxychlor (Martyniuk et al. 2011) and vinclozolin (Martinović et al. 2008; Smolinsky et al. 2010), suggesting that compounds found within the aqueous matrix have an anti-androgenic effect on fathead minnow larvae.

While the aqueous phase of the spring runoff was found to be anti-androgenic in minnow larvae, the harvested sediments elicited an anti-estrogenic effect. At 30 dph, significant downregulations in estrogen receptor α (*er α*) mRNA expression were detected between treatment groups (two-way ANOVA, $F=2.975$, $p=0.0457$; sediment source effect, $F=4.907$, $p=0.0342$) that were associated with the presence of the sediment matrix in the FWS and RWS aquaria (Figure 4.4). No differences in *er α* , or other

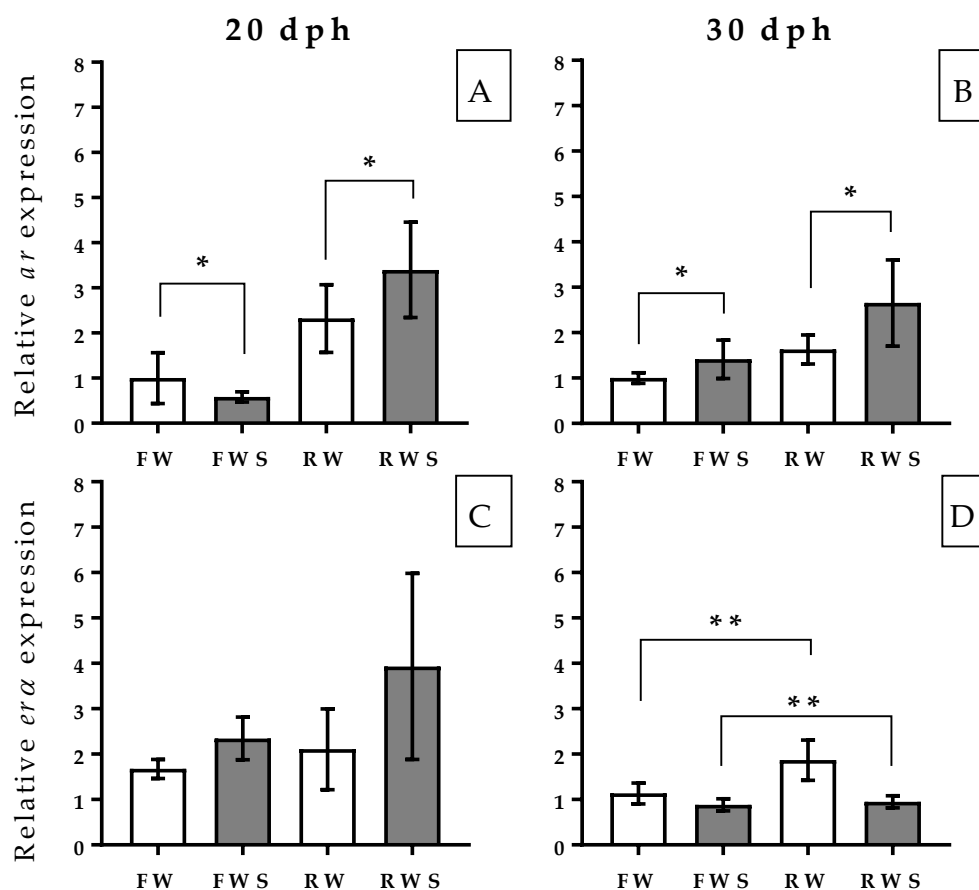


Figure 4. 4 Gene Expression Analysis of 2016 Minnow Larvae

Whole body relative gene expression results for 20-dph differences in androgen receptor (*ar*) mRNA (A) and estrogen receptor α mRNA (*era*) (C) along with 30-dph differences in *ar* mRNA (B) and *era* mRNA (D). Treatment groups on the x-axis are filtered water (FW), filtered water with sediment (FWS), river water (RW) and river water with sediment (RWS). Gene expression relative to ribosomal protein L8. Bars represent the mean (\pm SEM; $n=7-10$ per treatment group) of individual larvae from each treatment group. Significant differences detected by t-test are indicated by brackets with single asterisk for water type effect or double asterisks for sediment treatment effects ($\alpha=0.05$).

endocrine responsive genes, were detected in fathead minnow larvae at 20 dph in response to sediment treatment (Supplemental Table 6). Additionally, sediment exposure had a negative effect on body condition factor by 30 dph (two-way ANOVA, $F=7.020$, $p=0.0002$; sediment source effect, $F=12.51$, $p=0.0005$) which, as previously stated, was already reduced by exposure to river water. The anti-estrogenic effects of the Elkhorn's agriculturally contaminated sediments have been previously characterized through in vivo reductions of hepatic *era* mRNA expression in fathead minnows and in vitro reductions in estrogenic reporter activity of yeast cultures (Sellin Jeffries et al. 2011b). More recent evidence has shown that the anti-estrogenic effects in adult fathead minnows is associated with periods of increased river discharge that mobilize agrichemical-laden sediments (Ali and Kolok 2015; Zhang et al. 2015). Our results from the present study demonstrate that the anti-estrogenic effect of agrichemical-contaminated sediments is not exclusive to sexually mature minnows; rather, the sediment elicits anti-estrogenic effects across multiple life stages.

Combined, the matrix-specific effects on growth and gene expression identified in this study highlight the potential for exposure to both water and sediments found in agricultural runoff to affect early life stage fish. However, it is unclear whether these biological effects were due to the parent agrichemicals initially transported in runoff, their degradation and transformation within the environmental matrices, or other abiotic and biotic aspects of the runoff-derived water and sediments. Zhang et al. (2015) determined that the endocrine active effects of agricultural runoff in adult fish were tied to the mobilization and deposition of agrichemical-laden sediments rather than mere

occurrence of aqueous agrichemicals alone, suggesting an important role of the sediment matrix. Using sediments from the same location as Zhang et al. (2015) and the present study, Sangster et al. (2016) found that progesterone, a commonly detected steroid in manure-fertilized field runoff (Gall et al. 2011), preferentially adsorbs to sediments, drastically reducing its aqueous concentrations. As the supplemented progesterone degraded within the sediment, it produced aqueous androgens, namely testosterone and 4-androstenedione, leading to anti-estrogenic and androgenic effects in exposed female minnows (Sangster et al. 2016). Given this collective evidence, one plausible explanation for the matrix-specific effects in the present study is that the water effects were derived from hydrophilic agrichemicals carried during high flow events, whereas the sediment-specific effects were driven by more hydrophobic and sediment-associated contaminants released into the exposure systems. Additional studies are needed to determine whether the biological responses towards either matrix are due to a single agrichemical contaminant or the result of an uncharacterized mixture effect of multiple contaminants.

There are other potential sources for abiotic and biotic stressors in the water and sediments collected from Elkhorn River during the runoff periods. Sediment and water in this experiment were unsterilized, thereby making them biologically active matrices that can influence the fate and bioavailability of contaminants, nutrients (e.g. nitrates and organic carbon) and exposure to potential microbial pathogens. Additionally, the alkalinity, conductance and the concentrations of dissolved solids of river water treated aquaria were greater than those treated with laboratory water (Supplemental Table 5), which may have had subtle effects on growth and behavior of exposed larval (Pittman et

al. 2013; Kjelland et al. 2015). However, previous field studies at the same site in the Elkhorn River have not detected adverse biological responses during periods of reduced agrichemical concentrations associated with runoff despite the presence of suspended sediments and variation in other physicochemical parameters (Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015) suggesting the associated endocrine responses are the result of a yet unidentified causative agent carried by agricultural runoff.

Adult Impacts Following Early Life Exposure

Given the matrix specific effects on endocrine responsive genes in fathead minnow larvae, we expected organizational changes in the sex characteristics, reproductive performance and sex ratio of adult fish that reflected their early life exposure history. Upon reaching sexual maturity, male fathead minnows previously exposed to the water-sediment combination exhibited feminization of secondary sex characteristics as determined by an enlargement of their anal papillae relative unexposed males (two-way ANOVA, $F=5.090$, $p=0.0022$; interaction term, $F=6.747$, $p=0.0104$; Figure 4.5). No changes were detected in the relative ovipositor length of female fathead minnows exposed to any matrix found within the Elkhorn River's runoff (Supplemental Table 7). In female fathead minnows, this papillary structure is the ovipositor and its enlargement has been used as an indicator of the feminization of male fathead minnows following both adult and larval exposure to ethinylestradiol (Parrot and Blunt 2005; Salierno and Kane 2009).

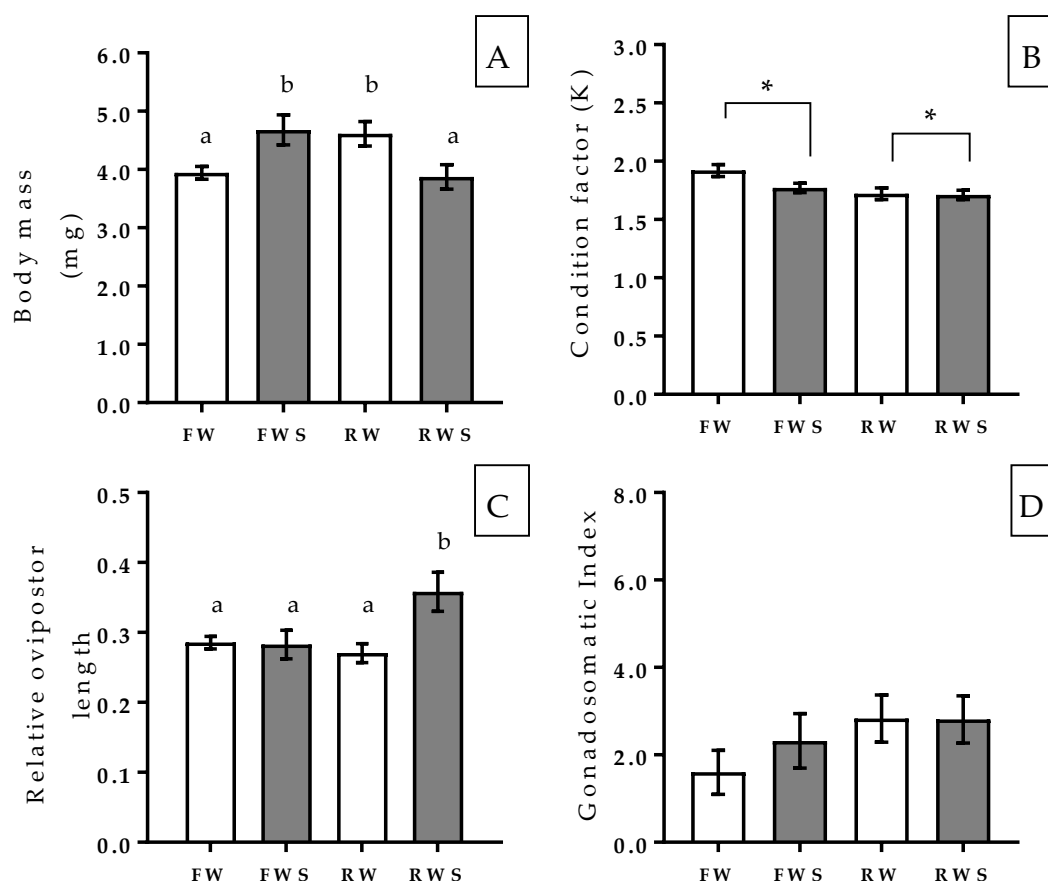


Figure 4.5 Morphometric Analysis for 2016 Adult Male Minnows

Body mass (A), condition factor (B) relative ovipositor length (C) and gonadosomatic index (D) of sexually mature male fathead minnows with prior larval exposure to filtered water (FW), filtered water with sediment (FWS), river water (RW) and river water with sediment (RWS) treatment groups. Bars represent the mean (± SEM; n=29-34 fish per treatment group) of each treatment group. Significant differences detected by Tukey's test for differences due to an interaction effect are denoted by lower case letters, whereas differences detected by t-test are indicated by brackets with single asterisk for water type effect or double asterisks for sediment treatment effects ($\alpha=0.05$).

Other than the presence of an ovipositor like structure in exposed males, there were no other persistent impacts in other sex characteristics including gonadosomatic index, number of tubercles, in either sex, or sex ratio based on larval treatment ($50.5 \pm 9.2\%$ female across all treatment groups). The breeding assay found no difference in the reproductive performance of adult fish due to their early life exposure to either water, sediment or water-sediment combination (Supplemental Tables 7 and 8).

Differences were also detected in the adult body masses and lengths of both sexes (Figure 4.4 and Supplemental Figure 3; Supplemental Table 7), but these changes did not reflect the same matrix-specific patterns observed in 20 or 30 dph minnow larvae. The only exception to this was the persistent reduction of body condition factor in adult males resulting from larval exposure to river water (two-way ANOVA, $F=4.085$, $p=0.0081$; water source effect, $F=4.880$, $p=0.0288$). While these interactive effects on adult growth were statistically significant, they are relatively subtle, making it difficult to clearly interpret without additional experiments that account for early life density-dependent growth effects, nutritional status, and social effects.

The results obtained from the depuration component of the present study were curious given the results from prior studies on the biological impacts of larval exposure to agricultural runoff (Leet et al. 2012; Ali et al. 2017). In May 2015, Ali et al. (2017) conducted an *in situ* exposure of fathead minnow larvae to a seasonal agricultural runoff event that initially suppressed growth and endocrine responsive gene expression, which was associated with increased concentrations of the same surrogate agrichemicals

detected in the present study. These effects in field exposed larvae were later compensated for following a two-week depuration under field conditions during a period of reduced agrichemical concentrations (Ali et al. 2017). In contrast, Leet et al. (2012) observed male-biased sex ratios by 40 dph following a longer exposure (50 d) to agricultural ditch water containing agrichemicals including steroids and pesticides, demonstrating a lack of recovery from early life exposure. The present study found that depuration under a best-case scenario allowed recovery in exposed minnows, with no untoward effects on sex ratios and reproductive performance. Collectively, these studies provide evidence that recovery is possible under certain conditions and is facilitated by compensatory responses of fathead minnow larvae, and that the compensation observed by Ali et al. (2017) likely has a limited capacity to reverse the effects of sustained exposure to endocrine-active agrichemicals described by Leet et al. (2012). Given that most contemporary aquatic environments experience some degree of background pollution, this finding raises important questions related to the roles of dose and duration in the compensatory response of minnow larvae and whether there a tipping point for recovery from early-life exposures.

Conclusions

The biological impacts in larvae and adult minnows following early life exposure to agricultural runoff collected from the Elkhorn River are summarized in Table 4.2. Minnow larvae exhibited matrix-specific alterations in growth and endocrine responsive

Table 4. 2 Summary of 2016 Exposure Study

Summary of biological impacts from exposure to treatment combinations relative to filtered water (FW) controls.

		River water (RW)	River water with sediment (RWS)	Filtered water with sediment (LWS)
Larval Minnow Responses	Morphometrics	↑ body mass ↓ condition factor	↓ condition factor	↓ condition factor
	Survival	no effect	no effect	no effect
	Gene Expression	anti-androgenic	anti-androgenic anti-estrogenic	anti-estrogenic
Adult Minnow Responses	Morphometrics	↑ ♂ body mass ↑ ♂ body length ↓ ♂ condition factor	↑ ♀ body length ↓ ♂ condition factor	↑ ♂ body mass
	Secondary Sex Characteristics ^a	no effect	ovipositor induction (♂)	no effect
	Primary Sex Characteristics ^b	no effect	no effect	no effect
	Reproductive Fitness ^c	no effect	no effect	no effect

^a Tubercle count and relative ovipositor length

^b Sex ratios and gonadosomatic index

^c Including fecundity, fertilization and cumulative egg production

gene expression that were consistent with previous studies of larval fish exposed to seasonal runoff. These larval effects did not translate into observable permanent effects upon reaching sexual maturity suggesting that fathead minnows can recover from an early life exposure under more optimal conditions. It is likely that the duration of early life exposure to the environmental matrices and subsequent depuration period played an important role in the recovery observed as adult minnows. An alternative explanation is that the window of sexual differentiation is not as susceptible to persistent effects as other windows of developmental programming such as the period of DNA methylation and epigenetic imprinting which has only recently started to be characterized in the fathead minnow (Wood et al. 2016). Under naturally occurring conditions, the contaminated sediments of the Elkhorn River act as a protracted source for agrichemicals (Zhang et al. 2015; Ali et al. 2017) making the post-exposure depuration in the present study a best-case recovery scenario. Furthermore, there is a need to identify whether the agrichemical mixture of the Elkhorn River quantified in this and other studies (Sellin et al. 2010; Sellin Jeffries et al. 2011b; Knight et al. 2013; Kolok et al. 2014; Ali and Kolok 2015; Zhang et al. 2015; Ali et al. 2017) is responsible for the observed biological effects or is merely a proxy for a yet unidentified causative agent. The dynamic interaction between agricultural runoff and its impacts on early life stage fish remains open for future investigation.

CHAPTER 5: ESTROGENIC EFFECTS FOLLOWING LARVAL EXPOSURE TO THE
PUTATIVE ANTI-ESTROGEN, FULVESTRANT, IN THE FATHEAD MINNOW
(*PIMEPHALES PROMELAS*).

The material in this chapter has been previously published: Ali J. M., Palandri M., Kallenbach A. T., Chavez E., Ramirez J., Onanong S., Snow D. D. & A. S. Kolok. (*in press*) Estrogenic effects following early-life exposure to the putative anti-estrogen, fulvestrant, in the fathead minnow (*Pimephales promelas*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*.

Introduction

There is a robust literature relative to the effects of pharmacological agents on the feminization of fish. Aqueous exposure of male fish to the synthetic estrogen 17 α -ethinylestradiol (EE2) induces the production of vitellogenin (*vtg*), an egg-yolk precursor protein, that is otherwise only produced naturally by female fish. This has been documented in several species including zebrafish (*Danio rerio*) (Martyniuk et al. 2007; Henry et al. 2009), fathead minnows (*Pimephales promelas*) (Pawlowski et al. 2004; Shappell et al. 2010; Armstrong et al. 2016), Japanese medaka (*Oryzias latipes*) (Chikae et al. 2004), brown trout (*Salmo trutta*) (Bjerregaard et al. 2008) and Murray rainbowfish (*Melanotaenia fluviatilis*) (Woods and Kumar 2011). There is evidence that the same concentrations of EE2 that induce *vtg* production also impair a number of other reproductive metrics, including: male fertility (Parrott and Blunt 2005), egg production (Armstrong et al. 2016), spawning behaviors (Majewski et al. 2002) and sex ratio to the point of population collapse (Kidd et al. 2007). Thus, feminization by estrogen agonists adversely impacts the health and reproduction of fish.

Although there is abundant information on the adverse impacts of feminization by estrogen agonists, there is a relative paucity of knowledge regarding the impacts of defeminization by estrogen antagonists. This is curious given the repeated observations of defeminization (Sellin Jeffries et al. 2011; Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015; Ali et al. 2017) as evident by the suppression of *vtg* and estrogen receptor- α (*era*) mRNA expression in female fish following exposure to agricultural

runoff under natural conditions. Using in vitro assays, Sellin Jeffries et al. (2011) demonstrated that this defeminization by an agrichemical mixture was mediated by antagonism of the estrogen receptor.

Defeminization mediated through the estrogen receptor is different from other pharmacological models of defeminization that primarily rely on inhibitors of aromatase (cyp19a, and cyp19b), the enzyme responsible for catalyzing the conversion of androgens into estrogens (reviewed by Cheshenko et al. 2008). Aromatase inhibitors, such as fadrozole, reduce the levels of circulating estrogens, thereby producing a defeminizing effect in female fish. However, this also has the unintended side effect of masculinization through elevated production of androgens (Villeneuve et al. 2009). While aromatase inhibitors have proven to be useful models for defeminization accentuated by masculinization of female fish, they do not provide insight into the effects of an estrogen antagonist devoid of androgenic effects.

A select few pharmacological estrogen antagonists have been used for in vivo experiments in fish. Tamoxifen demonstrates anti-estrogenic activity in zebrafish relative to the estrogenic effects of EE2 (Van Der Ven et al. 2007), but also elicits estrogenic responses such as *vtg* induction in male medaka (Chikae et al. 2004) and male fathead minnows (Williams et al. 2007). An alternative antagonist is ICI 189,154 which does not upregulate estrogenic gene expression in fathead minnows but is also incapable of inhibiting the effect of EE2 in co-exposure experiments (Garcia-Reyero et al. 2009;

Johns et al. 2011). Because of these conflicting results, there remains a need to identify a pure pharmacological estrogen antagonist for use in in vivo fish studies.

One plausible candidate is fulvestrant, also known as ICI 182,780 or Falsodex, which is a well characterized and potent estrogen-receptor antagonist in mammalian models (Hermenegildo and Cano 2000; Osborne et al. 2004). Studies utilizing recombinant whole-cell systems have demonstrated that the $er\alpha$ of the fathead minnow has an approximately 3x stronger binding affinity for fulvestrant than that of the human $ER\alpha$ (Rider et al. 2009). In vitro studies have shown that fulvestrant inhibits estrogen-responsive gene expression and protein production of *vtg* in hepatocytes from several species including: channel catfish (*Ictalurus punctatus*) (Monteverdi and Di Guilio 1999), Atlantic salmon (*Salmo salar*) (Celius et al. 1999), Siberian sturgeon (*Acipenser baeri*) (Lantonnette et al. 2002), largemouth bass (*Micropterus salmoides*) (Sabo-Attwood et al. 2007), brown trout (Madureira et al. 2015) and spotted scat (*Scatophagus argus*) (Cui et al. 2017). Despite in vitro evidence that fulvestrant acts as an estrogen-receptor antagonist in fish, fulvestrant has not been utilized to explore the effects of early-life suppression of estrogen-responsive genes in an environmentally-relevant model species such as the fathead minnow.

The objective of the present study was to investigate the consequences of early-life exposure to fulvestrant on estrogenic gene expression in fathead minnow larvae. To address our objective, fathead minnow larvae were exposed to fulvestrant during the window of sexual differentiation from 0 to 30 days post-hatch (dph) then assessed for

differences in endocrine-responsive gene expression, body size and survival. It was hypothesized that the expression of estrogen-responsive genes would be downregulated by exposure to fulvestrant. Following their early-life exposure, minnow larvae were transferred to clean water conditions to depurate until reaching sexual maturity, at which time they were assessed for persistent effects on gonadal development, sex characteristics, reproductive performance and adult sex ratio.

Materials and Methods

Animal Production and Maintenance

Fathead minnow larvae (fhml) used for this experiment were obtained from the Aquatic Toxicology Laboratory (ATL) at the University of Nebraska at Omaha, Omaha, NE. All procedures were conducted in compliance with protocols approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (Protocol #98-075-11) following previously described methods for breeding and maintenance (Ali et al. 2017). Adult fish were maintained in carbon-filtered dechlorinated tap water (filtered water) at 25.0 ± 1.0 °C with static 1/3 renewal of water once daily. All fish older than 30-days post-hatch (dph) were fed Tetramin Flake Food (Melle, Germany), whereas fhml (0-30 dph) were fed daily with a mixture of < 24 h old brine shrimp (*Artemia nauplii*) (INVE Aquaculture, UT, USA). Beginning on January 2, 2016, breeding triads of adult fathead minnows were established consisting of one male and two females. Fish were housed in 30 L aquaria, divided into two compartments by a

plastic, porous divider with two triads in each aquarium. Breeding triads were provided with a 12-cm half-length of 8 cm diameter PVC tubing as a breeding tile on which eggs were laid.

After the triads began to breed, the tiles with eggs were removed daily from the aquaria and transferred to 1 L aerated beakers. Unfertilized and fungus infected eggs were removed daily and surviving embryos were all hatched by 5-days post fertilization. Hatched fhml were transferred to 1 L beakers at a density of 100 larvae per liter and transferred to their respective exposure beakers.

Solution Preparation and Chemical Analysis

Fulvestrant (MW 606.77; CAS No. 129453-61-8) was acquired from Selleckchem and stored at -20 °C prior to stock solution preparation. A stock solution of 100 $\mu\text{g}\cdot\text{mL}^{-1}$, fulvestrant in ethanol, was prepared every 4 days and subsequently diluted in filtered laboratory water to achieve the nominal concentrations of 0.1 $\mu\text{g}\cdot\text{L}^{-1}$ and 10.0 $\mu\text{g}\cdot\text{L}^{-1}$ for the low-dose and high-dose groups, respectively. The stock solution was stored at 4 °C which has previously been reported to maintain stable concentrations of fulvestrant for up to 7-d (Clubbs and Brooks 2007). The final dilutions were used for once daily exchanges of water from the larval exposure beakers at a volume of 85%. Solvent controls were treated with filtered water containing 0.1% ethanol. Approximately 1 L water samples were collected from the low-dose and high-dose treatment groups on days 0, 10 and 20 and stored in glass amber bottles at -20 °C until further analysis.

To measure the concentrations of fulvestrant in aqueous solution, water samples were extracted and analyzed at the Water Sciences Laboratory at the University of Nebraska-Lincoln. Calibration solutions were prepared with analyte and surrogate concentrations at 0.5, 1, 5, 20, 50, and 100 $\mu\text{g}\cdot\text{L}^{-1}$ and internal standard concentration at 50 $\mu\text{g}\cdot\text{L}^{-1}$. A 100 mL portion of sample was measured and fortified with 25 ng of surrogate (a-Methyltestosterone) prior to solid phase extraction (SPE) with Oasis 200 mg HLB sorbent. Cartridges were conditioned with 5 mL of high purity methanol and 5 mL of purified reagent water, respectively. Cartridges were eluted with 5 mL of 0.1%v/v formic acid in methanol, followed by 5 mL of 0.1%v/v formic acid in acetonitrile, and the eluates were evaporated to dryness under vacuum and constant stream of nitrogen gas. Residues were fortified with 25 ng of internal standard (d5-testosterone) and reconstituted with methanol and water (50:50). A validation experiment using 8- 100 mL portions of a 0.10 ng/mL fortified blank provided a method detection limit ($\text{MDL} = \text{stn}-1$) of 0.067 $\text{ng}\cdot\text{mL}^{-1}$ with an average recovery of 62%. A fortified blank 1 $\text{ng}\cdot\text{mL}^{-1}$ was analyzed with the samples and recovery was 95%, and surrogate recovery averaged 79% in the samples.

Extracts were analyzed using multiple reaction monitoring (MRM) using liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Quattro Micro triple quadrupole mass spectrometer interfaced with a Waters 2695 HPLC. Analytes were monitored with atmospheric pressure photoelectron ionization (APPI) source in positive mode using toluene/methanol mixture (1:4) as dopant. An end-capped BetaBasic C18 reverse phase HPLC column (250x2 mm) was employed for gradient separation of

analytes at the flow rate of 0.3 mL/min. The gradient consisted of mobile phase A (0.15% v/v formic acid in 97:3 methanol/water) and mobile phase B (0.15% v/v formic acid in 97:3 water/methanol), with 0-1 min at 5%A, 1-3 min at 50%A, 3-14 min linear increased to 75%A, 14-20 min at 100% and back to 5%A for 10 min (30 min total analysis time). MRM transition for fulvestrant was 607.20 > 589. Further instrumental conditions are published elsewhere (Snow et al. 2013).

Larval Exposure to Fulvestrant

To investigate the effects of early life exposure to fulvestrant, fhml were exposed to fulvestrant from 0 to 30 dph (Figure 5.1). This exposure window corresponds to the window of sexual differentiation in the fathead minnow (van Aerle et al. 2004; Johns et al. 2009; Leet et al. 2013) and is consistent with standard early-life stage fish toxicity tests (OECD 1992). Minnow larvae were exposed to one of four treatment groups that included: filtered water control, solvent control (0.1% ethanol), low dose fulvestrant or high-dose fulvestrant.

At the start of the exposure, fhml (n=100 per replicate beaker) were transferred to 16 2-L glass Pyrex beakers supplied with filtered water and constant aeration. This provided 4 replicate beakers for each treatment group at the start of the exposure. Photoperiod was controlled at 16:8 h light:dark, and 85% of the water within each beaker was renewed daily with dilutions prepared for each respective treatment. Water quality parameters including temperature (°C), pH, conductivity (mS·cm⁻¹), and alkalinity (ppm)

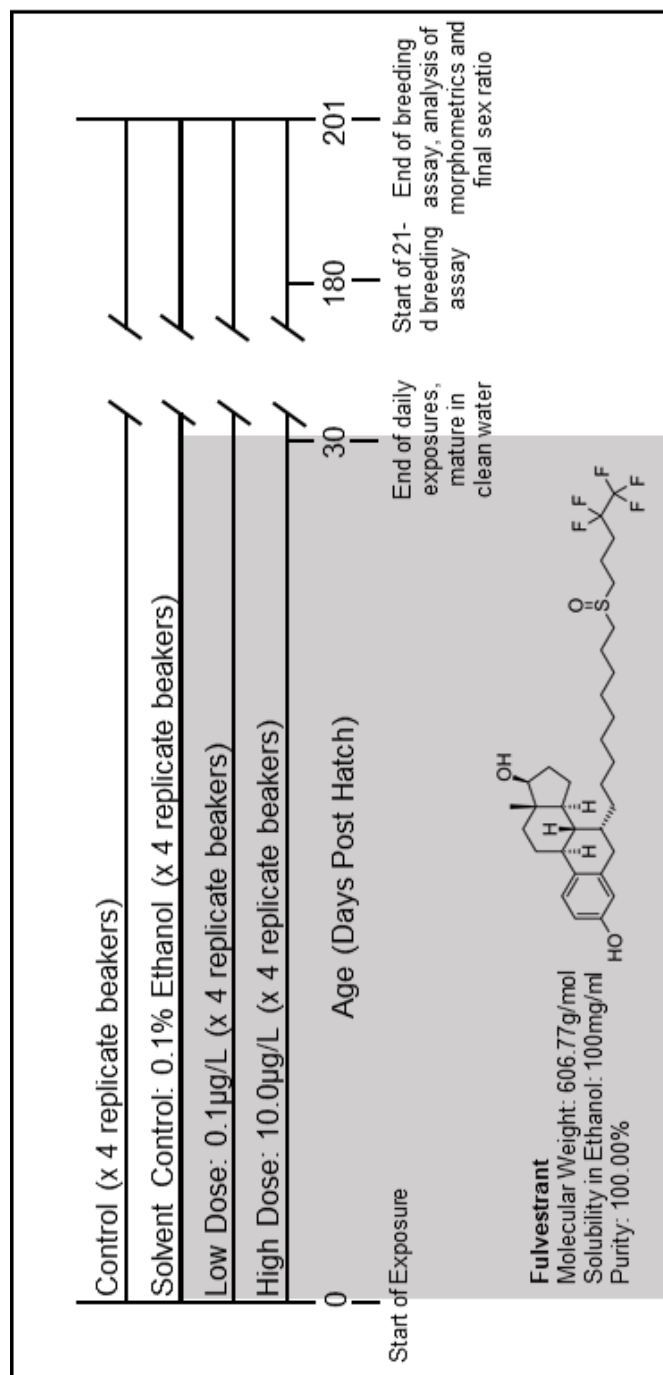


Figure 5. 1 Experimental Design for Exposure to Fulvestrant

Timeline for early-life exposure of fathead minnow larvae to fulvestrant. Shaded region corresponds to fulvestrant exposure in the low and high dose treatment groups. The exposure began with 100 fathead minnow larvae in each replicate beaker.

were measured daily throughout the 30-d exposure. Following the 30-d early life exposure, all remaining fhml were transferred to 30 L aquaria containing filtered laboratory water while continuously thinning their densities until reaching adulthood.

Larval Sampling and Gene Expression Analysis

On day 30 of the exposure, 15-20 larvae were randomly selected from each replicate beaker and sacrificed using a lethal dose of MS222 to collect morphometric measurements for body mass, body length and condition factor (K). From each of the 4 treatment groups, 15 of the sampled larvae were then immediately flash frozen and stored at -80 °C for further assessment by gene expression analysis. Survival was assessed at the end of the exposure by counting the number of larvae remaining in each exposure replicate.

Whole larvae bodies were used for reverse transcription quantitative polymerase chain reaction (RT-qPCR). RNA extraction utilized the SV Total RNA Isolation System (Promega, Sunnyvale, CA, USA) following manufacturer's recommendations. RNA was resuspended and stored in nuclease-free water at -80 °C until analysis. Purity and concentration of RNA were assessed by Nanodrop (NanoDrop Technologies, Wilmington, DE, USA) based on optical densities at 260 nm/280 nm and 260 nm/230 nm, where degraded or poor-quality RNA isolates were excluded from further analysis. Total extracted RNA samples were diluted to 15 ng·μL⁻¹ in preparation for cDNA synthesis. First-strand cDNA synthesis was performed using iScript cDNA Synthesis Kit

(Bio-Rad, Hercules, California, USA) per the manufacturer's recommendations. All RT-qPCR reactions were performed on the CFX Connect Real-Time PCR Detection System using the iTaq Universal SYBR® Green Supermix, 2x concentration, (Bio-Rad) per the manufacturer's protocol. Briefly, 2 µL of diluted cDNA template was added to 20 µM forward and reverse primers in a 15-µL volume containing iTaq Supermix. Target genes involved in endocrine signaling and synthesis were selected for analysis (primer sequences and their sources are provided in Table 5.1), these included: androgen receptor (*ar*), insulin-like growth factor 1 (*igf1*), gonadal aromatase (*cyp19a*), vitellogenin (*vltg*), estrogen receptor α (*era*) and estrogen receptor β (*erβ*). The reference gene ribosomal protein L8 (*rpl8*) was used to normalize gene expression (Kolok et al. 2007; Ali et al. 2017).

Reproductive Performance Assay

Reproductive assessment of both sexes following early-life treatment was conducted using a modified version of the 21-d breeding assay (Ankley et al. 2001). All fish used for this assay were 180 dph in age, approximately 6 months. This consisted of breeding triads with two females and a single male housed in 30 L aquaria, divided into two compartments by a plastic, porous divider with two triads in each aquarium. Breeding triads were provided with a 12-cm half-length of 8 cm diameter PVC tubing as a breeding substrate. To assess the impact of early-life exposure on males, 16 breeding triads were established consisting of four males from each of the filtered water control,

Table 5. 1 Primer Sequences for PCR

Primers used for real-time polymerase chain reaction analysis with respective annealing temperature and NCBI ascension number for each gene.

Gene	Forward sequence 5' - 3'	Reverse sequence 5' - 3'	Temp (°C)	R ²
<i>ar a</i>	GTTTCCGTAACCTGC ATGTGG	CGCGCATTAGCGTTC TTGTA	60.9	0.972
<i>vtg a</i>	TATGCACGAGAAAA TCGCCAC	AGCATGACGACTTCA CGCAG	65.0	0.986
<i>cyp19a b</i>	GCGGCTCCAGATACT C	ACTCTCCAGAATGTT TAACC	55.0	0.970
<i>era a</i>	AGTGAGCAGTCAAG CCGTGTT	GGTCAGGTGGCATGC ATAAAG	62.5	0.975
<i>erβ c</i>	ACAGTGTTTGATGAG CTACG	GGAGTCCACCCACC ATATCGTG CAT	61.1	0.973
<i>igf1 d</i>	GGCAAAACTCCACG ATCCCTA	ATGTCCAGATATAGG TTTCTTTGCTG	61.4	0.998
<i>rpl8 a</i>	GCCCATGTCAAGCAC AGAAAA	ACGGAAAACCCACCT TAGCCAG	59.2	0.997

^a Kolok et al. 2007; ^b Johns et al. 2009; ^c Filby et al. 2007; ^d Beggel et al. 2012

solvent control, low-dose and high-dose treatment groups were paired with filtered water control females. Similarly, reproductive performance of previously exposed females was assessed with an additional 12 breeding triads consisting of 8 females from each of the early-life exposure treatments.

After a 7-day acclimation period, spawning events were recorded daily after 11:00 AM. Spawning events were calculated as the number of days each pair spawned a clutch of eggs in the 21-d period. Once per week, spawning substrates with adhered eggs were removed from each tank and placed in a 1-L beaker containing filtered water maintained in a warm water bath at 25.0 ± 1.1 °C to quantify fertilization success. Clutch size, the number of eggs on each substrate, was counted so that fecundity could be determined. Cumulative egg production was determined by the total number of eggs produced by each breeding triad over the 21-d period. Fertilization success (% eggs fertilized) was determined weekly by counting the number of eggs with eye spots at 96 h post-spawn.

Histological Analysis

Following the 21-d reproductive performance assay, male and female fish were sacrificed with MS222 and their gonadal tissues were collected for histological analysis. Wet tissue weights and morphometric endpoints were measured as described below (See Morphometric Analysis). Dissected ovarian and testicular tissue samples were fixed in 10% buffered formalin (VWR International) and stored at room temperature until

further tissue preparation. The fixed samples were dehydrated in a series of increasing ethanol concentrations (50%–75%–95%–100%–100%) followed by 2 consecutive xylene baths, each solvent bath lasting 2 hours. The tissues were then embedded in paraffin and sectioned into 5- μ m thick sections adhered to glass slides using Permount (Fisher Scientific). Tissue slides were stained using hematoxylin (Ricca) and eosin (Thermo Scientific) following previously described methods (Shappell et al. 2010). Scoring of tissues for gonadal abnormalities including testes-ova, ovarian atresia or testicular degeneration followed procedures outlined by Leino et al. (2005).

Adult Morphometric Analysis

All remaining fish were sacrificed to determine sex ratios for each replicate aquaria from the four treatment groups. A subset of these males and females from each treatment group were assessed for body weight, fork length, and gonad masses (wet weight) and K. Primary sex characteristics of adult fish were assessed through gonadosomatic index (GSI) where $GSI = ((\text{gonad mass (g)} / \text{body mass (g)}) \times 100)$ and final sex ratio (% female) as determined by visual inspection upon dissection. Secondary sex characteristics in both adult male and female minnows included tubercle counts (a marker of masculinization) and the ovipositor length (a female-specific external structure for egg laying and marker of feminization) relative to whole body length (Parrott and Blunt 2005; Jensen et al. 2001; Ankley et al. 2001).

Statistical Analysis

Data were analyzed using JMP 11 Software (SAS). All data were assessed for conformity to parametric assumptions, including equal variances by Levene's test. Morphometric data (n=72-82 per treatment group) and gene expression data (n=7-11 per treatment group) collected from 30-dph fhml were compared by one-way ANOVA across the four treatment groups. Where significant differences were detected, Tukey's test was performed. Data collected from adult male minnows including body mass, length, K, GSI, relative ovipositor length, tubercle count and reproductive assay results were compared by one-way ANOVA followed by Tukey's test. Similarly, data collected from adult female minnows were compared by one-way ANOVA followed by Tukey's test. Proportional data for final sex ratios (% female), larval survival and adult fertilization score were arcsine transformed to satisfy parametric assumptions for one-way ANOVA (Sokal and Rolf 1995). Histological data for the incidence of gonadal abnormalities were compared by Chi-square test. Statistical significance was assumed at $\alpha=0.05$.

Results

Water Chemistry

Water quality parameters throughout the experiment were as follows (mean \pm SEM): temperature 24.4 ± 0.1 °C, pH 8.04 ± 0.32 , specific conductance 0.417 ± 0.024 mS·cm⁻¹ and alkalinity 121.1 ± 3.0 ppm. Over the course of the 30-d larval exposure, the

mean (\pm SEM) aqueous concentrations of fulvestrant were $0.03 \pm 0.01 \mu\text{g}\cdot\text{L}^{-1}$ in the low-dose exposure beakers and $1.92 \pm 1.32 \mu\text{g}\cdot\text{L}^{-1}$ in the high-dose exposure beakers.

Larval Morphometrics and Survival

By 30 dph, the solvent control and low-dose exposed fhml exhibited a greater body condition factor than the filter water controls (Table 5.2), whereas the high-dose exposed fhml were not different from any group (ANOVA; $F_{3, 295} = 4.510$, $p = 0.0041$; Tukey's test). There were no differences detected between treatment groups with respect to larval body mass or length. No differences were detected in survival by 30 dph with a mean survival (\pm SEM) of each treatment group as follows: filter water control $72.5 \pm 5.1\%$, solvent control $71.4 \pm 11.1\%$, low-dose fulvestrant $74.5 \pm 7.8\%$ and high-dose fulvestrant $74.3 \pm 4.5\%$.

Larval Gene Expression

Figure 5.2 shows whole-body mRNA levels for the six genes of interest analyzed in 30 dph fhml. Relative to both controls, the low and high doses of fulvestrant upregulated the expression of the estrogenic genes *er β* (ANOVA; $F_{3, 34} = 4.941$; $p = 0.0059$; Tukey's test) and *vtg* (ANOVA; $F_{3, 32} = 7.666$; $p = 0.0005$; Tukey's test). Additionally, low and high doses of fulvestrant upregulated the expression of *ar* relative to both filtered water and solvent controls (ANOVA; $F_{3, 33} = 4.205$; $p = 0.0126$; Tukey's test). No differences were detected in the relative gene expression of *era*, *igf1* or *cyp19a*.

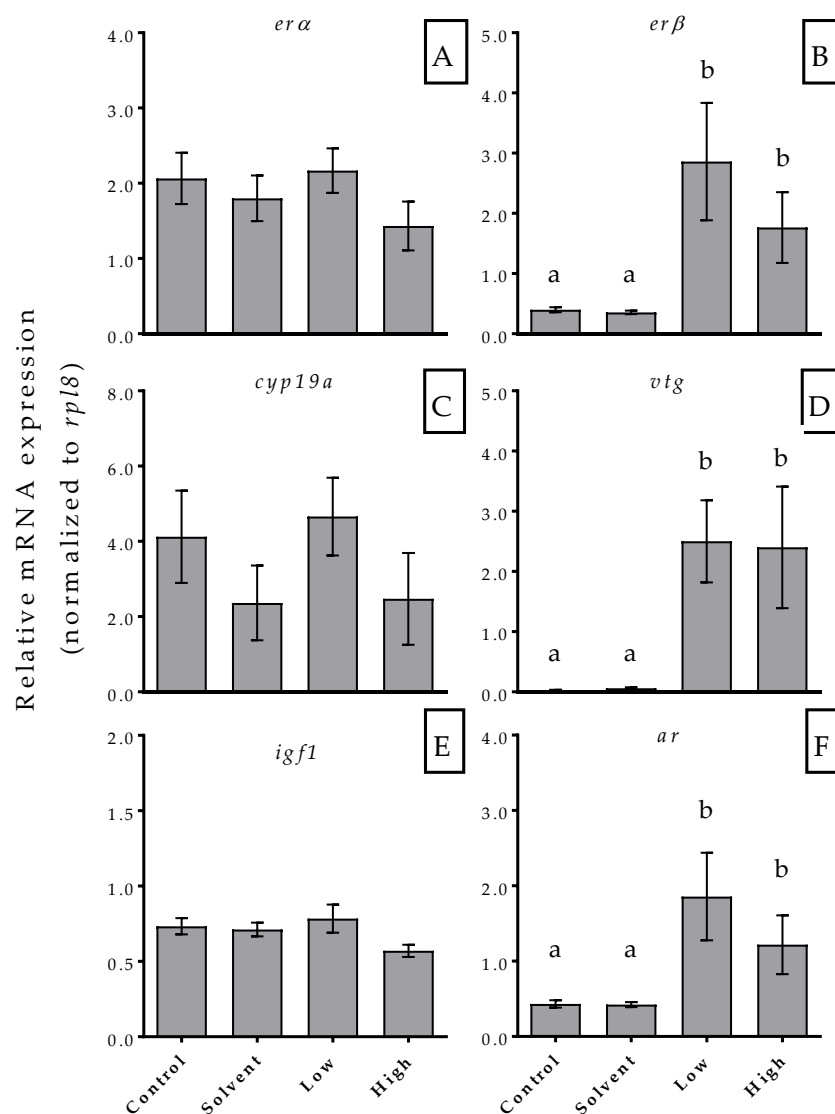


Figure 5. 2 Gene Expression Analysis from Larval Exposure to Fulvestrant

Relative gene expression for fathead minnow larvae following 30 d exposure to their respective treatment groups. Genes of interest include estrogen receptor α (*era*, A), estrogen receptor β (*erβ*, B), gonadal aromatase (*cyp19a*, C), vitellogenin (*vtg*, D), insulin-like growth factor 1 (*igf1*, E) and androgen receptor (*ar*, F). Treatment groups include filter water control (Control), 0.1% ethanol solvent control (Solvent), low-dose fulvestrant (Low) and high-dose fulvestrant (High). Significant differences detected by one-way ANOVA are indicated by lower case letters (Tukey's test; $p < 0.05$) with a sample size of 7-10 larvae per treatment group. Bars represent the mean \pm SEM.

Table 5. 2 Body Mass and Length of Larval and Adult Minnows

Body mass, length and body condition factor of fathead minnow larvae and sexually mature adults. Values presented as mean \pm SEM.

	Control	Solvent	Low	High
Minnow Larvae (30 dph) ^a				
Body mass (mg)	68.5 \pm 3.61	78.3 \pm 3.64	81.2 \pm 3.64	76.1 \pm 3.64
Body length (mm)	16.6 \pm 0.26	16.8 \pm 0.26	17.0 \pm 0.26	16.8 \pm 0.26
Condition factor	1.44 \pm 0.04 a	1.60 \pm 0.04 b	1.62 \pm 0.04 b	1.53 \pm 0.04 ab
Adult Females (201 dph) ^b				
Body mass (g)	1.80 \pm 0.06	1.83 \pm 0.06	1.76 \pm 0.06	1.71 \pm 0.06
Body length (cm)	4.91 \pm 0.07	4.98 \pm 0.06	4.94 \pm 0.07	4.78 \pm 0.07
Condition factor	1.54 \pm 0.04	1.48 \pm 0.03	1.44 \pm 0.04	1.56 \pm 0.04
Adult Males (201 dph) ^c				
Body mass (g)	3.02 \pm 0.14	3.09 \pm 0.13	2.94 \pm 0.13	3.11 \pm 0.10
Body length (cm)	5.77 \pm 0.12	5.98 \pm 0.12	5.88 \pm 0.11	6.03 \pm 0.09
Condition factor	1.51 \pm 0.04	1.45 \pm 0.08	1.40 \pm 0.04	1.39 \pm 0.03

^a n=72-73 per treatment group

^b n=88-103 per treatment group

^c n=72-97 per treatment group

Adult Morphometrics and Sex Characteristics

No significant differences were detected between treatment groups with respect to the adult body mass, fork length or body condition factor of either male or female fathead minnows (Table 5.2). Additionally, no differences were detected in sex ratios upon reaching sexual maturity with the mean percentage of females (\pm SEM) of each treatment group as follows (n=4 replicate tanks per treatment group): filter water control $53.3 \pm 6.5\%$, solvent control $59.5 \pm 10.1\%$, low-dose fulvestrant $47.1 \pm 10.6\%$ and high-dose fulvestrant $47.8 \pm 9.3\%$.

Figure 5.3 presents the morphological sex characteristics of both male and female fish following early-life exposure to their respective treatment groups. In male fathead minnows, prior exposure to fulvestrant resulted in a 48% and 55% reduction in relative testicle size (GSI) in comparison to those exposed to filtered water alone (n=72-97; ANOVA; $F_{3, 342}=7.427$, $p<0.0001$; Tukey's test). Early life exposure to the high-dose fulvestrant treatment led to the induction of an ovipositor-like structure at the anal pore that was nearly 20% longer than that of sexually-mature filter water treated male minnows (n=57-72; ANOVA; $F_{3, 257}=3.274$, $p=0.0217$, Tukey post hoc). For the average tubercle count, there was a subtle effect of early-life treatment (ANOVA; n=57-72; $F_{3, 257}=2.739$, $p=0.0439$), however, Tukey's test failed to discern a difference between any treatment group.

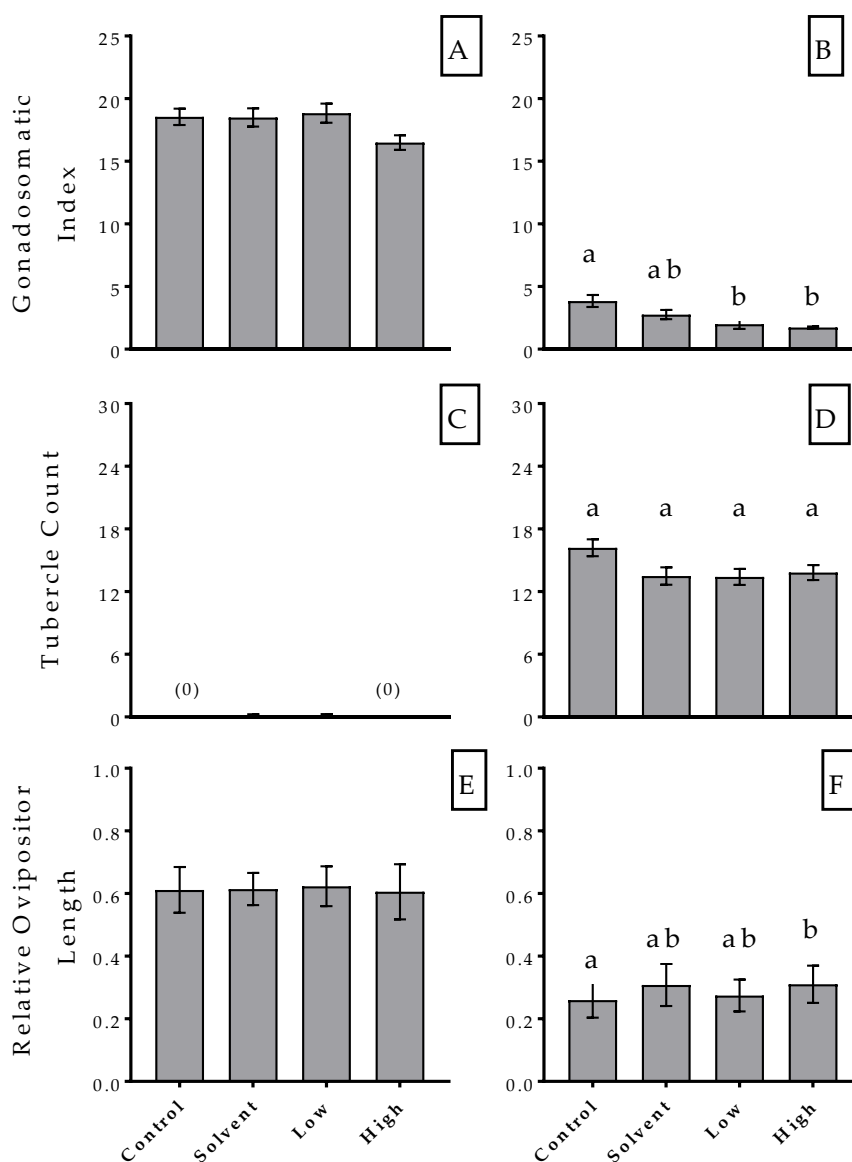


Figure 5.3 Sex Characteristics Following Depuration from Fulvestrant

Gonadosomatic index (GSI) (A,B), tubercle counts (C,D) and relative ovipositor length (E, F) for sexually mature female (A,C,E) and male (B,D,F) fathead minnows following early-life exposure to fulvestrant. Treatment groups include filter water control (Control), 0.1% ethanol solvent control (Solvent), low-dose fulvestrant (Low) and high-dose fulvestrant (High). Significant differences detected by one-way ANOVA are indicated by lower case letters (Tukey's test; $p < 0.05$) with sample sizes between 57-92 (as detailed in Results). Bars represent the mean \pm SEM.

No significant differences were detected in the relative ovary size (GSI), ovipositor length or tubercle counts of the female minnows in response to early life treatment (Figure 5.3; n=68-72 per treatment group).

Adult Reproductive Performance

Following the 21-d breeding assay, no significant differences were detected in cumulative egg production, clutch size, number of spawning events or fertilization success in the treated male or treated female breeding triads (Table 5.3).

Adult Gonadal Histology

Histological assessment of ovaries and testes did not detect any significant differences between the treatment groups relative to filtered water controls following the reproductive assay (data not shown). Similarly, histological assessment of the testes from males in the reproductive assay did not detect the presence of ova-testes.

Discussion

The objective of the present study was to investigate the consequences of early-life exposure to fulvestrant on estrogenic gene expression in fathead minnow larvae. This was accomplished by assessing estrogenic gene markers in fathead minnow larvae following a 30-d early life exposure to the putative anti-estrogen, fulvestrant. The

Table 5. 3 Breeding Assay Result Following Early-life Exposure to Fulvestrant
Results from reproductive assay of treated male and female fish paired with control fish of the opposite sex. All values represent the mean \pm SEM.

	Control Males with Treated Females			
	Control ♀	Solvent ♀	Low ♀	High ♀
Cumulative Egg Production ^a	3507 \pm 485	3352 \pm 452	3062 \pm 485	3230 \pm 50
# Spawning Events ^a	12.3 \pm 0.9	14.0 \pm 1.1	13.0 \pm 1.8	11.3 \pm 0.5
Average Clutch Size ^a	287.2 \pm 33.4	241.0 \pm 29.1	234.2 \pm 9.8	288.3 \pm 11.7
Fertilization (%) ^b	84.6 \pm 7.0	97.8 \pm 5.7	79.4 \pm 7.7	82.0 \pm 8.6
	Treated Males with Control Females			
	Control ♂	Solvent ♂	Low ♂	High ♂
Cumulative Egg Production ^a	3507 \pm 472	4135 \pm 645	2817 \pm 669	2459 \pm 582
# Spawning Events ^a	12.3 \pm 0.9	12.5 \pm 1.8	10.3 \pm 1.3	11.5 \pm 2.2
Average Clutch Size ^a	287.2 \pm 33.4	366.6 \pm 108.6	268.5 \pm 34.1	221.4 \pm 45.5
Fertilization (%) ^c	84.6 \pm 7.0	82.9 \pm 7.2	79.3 \pm 10.2	87.2 \pm 8.6

^a n=4 per treatment group

^b n=4-9 per treatment group

^c n=5-10 per treatment group

outcomes from this study indicate that aqueous exposure to fulvestrant produces estrogenic effects in fathead minnow larvae at the macromolecular level, as well as persistently feminizing and demasculinizing the adult sex characteristics of male fathead minnows.

Aqueous Exposure to Fulvestrant

To our knowledge, this is the first study in which an aquatic organism was exposed to fulvestrant and the aqueous concentration of fulvestrant in the water was directly measured. The aqueous concentrations of fulvestrant were on average only 30% and 28.6% that of the nominal concentrations for the low and high dose treatment groups, respectively. Although this indicates that the exposure was below the desired concentrations, the 95.3-fold difference between the low and high-dose treatments was consistent with the nominal difference in concentrations between these groups. One likely explanation for the reduced aqueous concentrations is the sorption of fulvestrant to organic material (e.g. feces or food particles) within the exposure beaker given its relatively hydrophobic log K_{ow} that has been estimated between 7.67-9.09 (US EPA 2004; Poulin and Haddad 2012).

There is relatively little information on the aqueous stability of fulvestrant under similar conditions as those maintained in the present study. Unlike the present study, other studies conducting aqueous exposures of non-piscine species such as frogs (Mackenzie et al. 2003) or invertebrates (Clubbs and Brooks 2007; Bannister et al. 2013)

omitted measuring aqueous concentrations of fulvestrant. While the present study provides a method for measurement of aqueous fulvestrant, additional studies are required to characterize the stability and metabolite profile of fulvestrant in aquatic systems.

In Vivo Estrogenic Effects of Fulvestrant on Fishes

The present study provides evidence that aqueous exposure to fulvestrant elicits both estrogenic and anti-androgenic responses in fathead minnow larvae. This was determined by the upregulation of estrogenic genes including *vtg* and *erβ* (Figure 5.2). This was unexpected, as similar upregulations of the same genes, especially *vtg*, have been consistently reported in response to estrogens such as EE2 (Panter et al. 2002; Biales et al. 2007; Johns et al. 2011) and estrone (Dammann et al. 2011). Had fulvestrant inhibited estrogen receptor activity as was expected, we would have observed either a downregulation or no difference in the expression of estrogen-responsive genes relative to their controls. In addition to estrogenic effects, fulvestrant also elicited anti-androgenic effects. The effects were consistent with the effect of anti-androgens, such as vinclozolin or flutamide, on fathead minnows, as they elicit an upregulation of *ar* expression (Filby et al. 2007; Martinovic et al. 2008).

We are only aware of one other study in which fish were exposed in vivo to fulvestrant. Pinto et al. (2006) investigated the use of fulvestrant as a potential estrogen antagonist in adult male sea bream (*Sparus auratus*) using intraperitoneal injections of

fulvestrant and 17 β -estradiol. Sea bream injected with high doses of fulvestrant (10 mg·kg⁻¹) exhibited upregulations in hepatic *era* and *vtg* gene expression, without inhibiting the estrogenic effects of 17 β -estradiol (1 mg·kg⁻¹) co-administered with fulvestrant (4 mg·kg⁻¹) (Pinto et al. 2006). Interestingly, the effects of 17 β -estradiol on hepatic gene expression were potentiated by administration of fulvestrant 3 d prior to the administration of 17 β -estradiol. The estrogenic effects produced through this parenteral route of administration provides evidence that, at least in sea bream, the mechanism for estrogen agonism by fulvestrant is a direct interaction of the molecule with the receptor site (Pinto et al. 2006).

Disparity Between In Vivo and In Vitro Experiments

While the two in vivo studies above demonstrate estrogenic effects, in vitro studies in fish and mammalian models have demonstrated the anti-estrogenic activity of fulvestrant (Monteverdi and Di Guillo 1999; Celius et al. 1999; Lantonnelle et al. 2002; Sabo-Attwood et al. 2007; Madureira et al. 2015; Cui et al. 2017). This would suggest that the differential responses between in vitro and in vivo systems are the result of either i) the transformation of fulvestrant to an estrogenic metabolite in larval minnows or ii) species-specific differences in the estrogen receptor's binding affinity for fulvestrant. One plausible explanation for the disparity between the in vitro and in vivo studies is that fulvestrant was metabolized within the tissues of the minnow larvae, thereby producing estrogenic daughter-metabolites. Human and rodent studies have shown that

fulvestrant is readily metabolized by cytochrome p450 enzymes (i.e., CYP3A4) yielding ketone, sulphate, sulphone and glucuronide metabolites (Robertson and Harrison 2004); yet none of these metabolites display any observable estrogenic activity in in vitro mammalian models. However, the metabolism of fulvestrant in fish is poorly characterized, leaving the production and potential effects of its daughter metabolites open to speculation.

An alternative explanation is that fulvestrant, in parent form, has estrogenic activity in fathead minnows at the concentrations used in the present study. Fulvestrant has a stronger binding affinity for $\text{er}\alpha$ in fathead minnows relative to humans (Rider et al. 2009), but it has not been determined whether this results in the activation or suppression of estrogen-responsive genes, such as *vtg*. In mammals, the side chain moiety of fulvestrant (Figure 5.1) affects the stability of the estrogen receptor which prevents dimerization and accelerates its degradation, thereby preventing the initiation of transcription at estrogen response elements found in DNA (Osborne et al. 2004). It is unknown whether fulvestrant can destabilize the estrogen receptors of fish in a similar fashion as reported in mammalian studies, however, the enhanced estrogenic activity observed in the present study suggests that it does not.

Effects of Larval Exposure as Adults

The feminization of minnow larvae by fulvestrant led to the demasculinization of sexually mature males as determined by reductions in the relative mass of their testes at

both exposure doses (Figure 5.3). This reduction in GSI was not associated with the any incidence of observable degeneration of testicular architecture or gonadal intersex (i.e., ova-testes) at the histological level, or any observable impacts in their reproductive performance (Table 5.3). In fact, the fertilization rate of the previously exposed males (79.3-87.2%) was consistent with prior reports of healthy male fathead minnows (between 81-82%; Parrot and Blunt 2005). Leino et al. (2004) showed that after a single spawning event the testicular tubules of the fathead minnow are not depleted of mature sperm, indicating that male minnows do not require the full contents of an intact teste to successfully fertilize a clutch of eggs. Furthermore, male fathead minnows do not experience significant changes in GSI over the course of a 21-d breeding period and continuously produce sperm so long as they are maintained under optimal conditions (Jensen et al. 2001; Hala et al. 2009). Therefore, the estrogenic effect of fulvestrant appears to have stunted the initial growth of the testes in exposed minnow larvae, resulting in an organizational impact that did not translate into an adverse outcome in adult reproductive performance.

The secondary sex characteristics of male minnows also showed signs of feminization due to early life exposure to fulvestrant. The ovipositor is a normal secondary-sex characteristic of female fathead minnows which is absent in males who only possess smaller anal papillae. Thus, the enlargement of the male anal papillae is considered a biomarker of feminization (Parrot and Blunt 2005). Only the males exposed to the highest dose of fulvestrant as larvae possessed enlarged anal papillae reminiscent of an ovipositor. A subtle effect of early-life treatment was detected in the number of

nuptial tubercles possessed by males, but post-hoc analysis could not discriminate any difference between treatment groups, making it impossible to interpret the effect of treatment on this marker of masculinization. Unlike the testes and ancillary reproductive organs that begin differentiation during the same timeframe as exposure to fulvestrant in the present study, nuptial tubercles do not develop until reaching sexual maturity and are under the positive control of androgens (Jensen et al. 2001). Unfortunately, serum 11-ketotestosterone levels were not measured, hindering our ability to link the subtle effect on nuptial tubercles with the more overt effects observed in the testes.

No alterations were observed in average ovary size, secondary sex characteristics or reproductive performance of female fish following early-life exposure to fulvestrant. Given the observed feminization of co-exposed male fish, it is reasonable to conclude that organizational effects were not observed in female fish because the estrogenic effects of fulvestrant were not antagonistic to the estrogenic cues required for differentiation and maintenance of the ovary.

Conclusions

Collectively, the results from this study provide evidence that aqueous exposure to the putative antiestrogen fulvestrant feminizes fathead minnows. This demonstrates the need for caution when selecting pharmacological agents as *in vivo* antagonists in fishes based on evidence collected from *in vitro* experiments. Furthermore, this

differential response to a putative estrogen antagonist underscores the complexity of estrogen receptor signaling and associated gene expression in fishes. Additional research is required to identify pure pharmacological estrogen antagonists so that we might better understand the adverse impacts of defeminization in fishes.

CHAPTER 6: DISCUSSION

The specific objective of this dissertation was to investigate the impact of early-life EDC-initiated induction and suppression of gene expression on organizational effects in the fathead minnow. It was hypothesized that the early-life induction of estrogen-responsive genes by endocrine disrupting compounds (EDCs) would lead to organizational effects, whereas the early-life suppression of these genes would not result in organizational effects. This was evaluated by a series of experiments that were designed to address the following three aims:

1. Determine whether episodic exposure to EDCs in early development results in adverse organizational impacts in the fathead minnow.
2. Determine whether early-life suppression of estrogen-responsive gene expression in fathead minnow larvae results in adverse organizational effects.
3. Determine whether early-life induction of estrogen-responsive gene expression in fathead minnow larvae results in adverse organizational effects.

Findings Relative to Aim 1.

Early-life exposure to EDCs produces sex-specific organizational effects in the fathead minnow. The experiments of Chapters 4 and 5 demonstrate that early-life exposure in the present study resulted in the organizational feminization (*i.e.*, ovipositor induction) and demasculinization (*i.e.*, reduced testes size) of male fathead minnows. However, female minnows that were co-exposed as larvae in the same experiments showed no observable organizational effects in their sex characteristics or reproductive performance. These

observations were curious, as gene expression analysis of minnow larvae identified biomarkers of anti-estrogenic and anti-androgenic effects following exposure to agrichemical runoff, and biomarkers of estrogenic and anti-androgenic effects following exposure to fulvestrant. Given the presumptive roles of androgens and estrogens in sexual differentiation of male and female fishes, respectively (Devlin and Nagahama 2002), it was expected that each sex would have experienced organizational effects from at least one of these exposures. However, only male fathead minnows experienced observable organizational effects following their early-life exposure.

The sex-specific susceptibility to organizational outcomes suggests that early sexual differentiation in male fathead minnows is more dependent on endocrine signals than sexual differentiation in female minnows. Other studies using fathead minnows have demonstrated that the onset of ovarian differentiation is followed by upregulations of *cyp19a* and *era*, all of which precedes the upregulation of *ar* and testicular differentiation in males by 10-20 days (Johns et al. 2009; Leet et al. 2013). There is evidence that the mechanism for sex determination in the fathead minnow is polygenic (Olmstead et al. 2011), where sex determination and differentiation are regulated by multiple genes across separate chromosomes that are expressed in a coordinated fashion resulting in each sex phenotype. If the onset of ovarian differentiation is more closely regulated by genetic factors than hormones, this might offer a plausible explanation for the absence of organizational effects in female minnows despite the suppression of estrogen signaling by agrichemicals in Chapter 4. Unfortunately, the exact mechanism for sex determination and differentiation of the fathead minnow remains

uncharacterized, thereby limiting our understanding of how the EDC exposures of the present study might have elicited their sex-specific organizational impacts. The findings of the present study beg the question as to whether sex determination and differentiation of both sexes in the fathead minnow are under equal control of genetic and environmental factors, and how this might influence susceptibility to organizational effects.

Although organizational outcomes were detected in the sex characteristics of male minnows, neither exposure described in Chapter 4 or 5 had an adverse impact on their reproductive performance (*i.e.*, fertilization success). As discussed in Chapter 5, the testes of the male minnow continuously produce sperm under optimal conditions and are not impaired by the reductions in testicular mass observed in the present study. Such aspects of reproductive biology are important considerations when evaluating organizational impacts as the physiology or reproductive strategy of a model species can allow compensation for subtle organizational effects induced by early-life insults.

Findings Relative to Aim 2.

Early-life suppression of estrogen-responsive gene expression is not associated with organizational effects as adults. The same agrichemical runoff that has been previously demonstrated to defeminize adult fish (Sellin et al. 2011; Knight et al. 2013; Ali and Kolok 2015; Zhang et al. 2015) elicited anti-estrogenic responses from exposed minnow larvae in the present study (Chapters 2 and 4). Curiously, the anti-estrogenic response of

larvae was not associated with any observable organizational effects as sexually-mature adults. This indicates that fathead minnow larvae can respond in a similar fashion as adult fish towards an anti-estrogenic mixture of agrichemicals, but these responses are activational and not organizational.

In addition to the anti-estrogenic response towards the spring agrichemical runoff, fathead minnow larvae experienced an anti-androgenic induction of *ar* following early-life exposure that was associated with adverse organizational impacts in male minnows. Following agrichemical exposure, male minnows possessed enlarge anal papillae reminiscent of a female's ovipositor (Chapter 4). Similarly, anti-androgenic effects following exposure to fulvestrant (Chapter 5) were also associated with the induction of an ovipositor as well as reduced testicular size in adult male minnows. Although the estrogenic effects of fulvestrant in the latter exposure certainly contributed to the feminization of male minnows, the organizational effects in both Chapters 4 and 5 were associated with the induction of androgen-responsive gene expression instead of the suppression of estrogen-responsive gene expression.

To our knowledge, this study is the first to associate anti-androgenic responses of fathead minnow larvae with organizational effects in adults. One other study evaluated the effects of exposing fathead minnow larvae to the anti-androgenic fungicide vinclozolin ($90\text{--}1,200\text{ }\mu\text{g}\cdot\text{L}^{-1}$) from 0-30 dph but found that this did not produce adverse impacts on growth, sexual differentiation or reproductive performance as adults (Makynen et al. 2000). However, the study by Makynen et al. (2000) did not quantify the

transcriptional responses of minnow larvae nor did they assess adult sex characteristics, making it difficult to compare with the anti-androgenic responses and organizational outcomes from minnows in the present study.

Another challenge is the interpretation of the up- or downregulation of ar expression and its associated genes (Chapters 3 and 4). The regulatory function of the androgen receptor and its expression in teleost fish is poorly characterized (Filby et al. 2007; Leet et al. 2011), requiring further investigation to better implement its use as a reliable biomarker of androgenic and anti-androgenic effects in both larval and adult fish.

Findings Relative to Aim 3.

Early-life induction of estrogen responsive genes contributes to organizational effects in male fathead minnows. Early-life exposure to fulvestrant induced estrogenic changes in the gene expression of larval fish that were followed by the feminization of the adult male sex characteristics. The organizational feminization of males is consistent with previous studies of utilizing lifelong exposures to potent estrogens such as ethinylestradiol (EE2) (Guillette et al. 1995; Länge et al. 2001; Van Aerle et al. 2002; Parrott and Blunt 2005); however, the organizational impacts observed in the present study were not associated with adverse impacts on reproductive performance as adults. Therefore, the combination of estrogenic and anti-androgenic activational changes in

gene expression in minnow larvae likely contributed to the induction of organizational impacts observed in Chapters 4 and 5.

Implication for the Paradigm of Activational and Organizational Effects

At the time of this dissertation, it has been twenty-two years since Guillette et al. (1995) argued that activational and organizational responses were central to interpreting the toxicity of EDCs. In that time, dozens of studies have characterized organizational effects following early-life exposure to EDCs, namely estrogenic substances (Länge et al. 2001; Van Aerle et al. 2002; Parrott and Blunt 2005; Kidd et al. 2007; Palace et al. 2009). These studies have led to the presumption that organizational responses from early-life exposures are inherently detrimental to the affected physiological system. This presumption has been extended beyond the realm of experimental physiology and toxicology by the US EPA's Adverse Outcome Pathway for the prediction of organismal level outcomes following molecular initiating events (Ankley et al. 2010; Villeneuve et al. 2013). However, the results of the present study demonstrate the need for caution in associating organizational effects with adverse outcomes at the organismal level.

Given the findings of this study, there are two plausible explanations for the lack of *adverse* organizational impacts observed following early-life induction of estrogen responsive genes. Either 1) early-life exposure to the anti-estrogenic, estrogenic and anti-androgenic compounds in the present study is not capable of producing adverse effects

on adult reproductive function, or 2) depuration following early-life exposure allows for compensation and recovery in the fathead minnow.

The first explanation is that the mechanisms of endocrine disruption observed in the present experiments do not elicit organizational responses that adversely affect adult reproductive performance. It is recognized that lifelong exposures to potent estrogen agonists result in adverse organizational impacts in various fishes (Guillette et al. 1995; Devlin and Nagahama 2002; Sumpter 2005). For example, Parrott and Blunt (2005) have shown that lifelong exposure to low concentrations of EE2 (0.32 and 0.96 ng·L⁻¹) feminized adult male fathead minnows and negatively affected their fertility. Other studies using anti-estrogens (Johns et al. 2011) have not been extended in a similar fashion as the previously mentioned EE2 studies to evaluate the potential for any organizational effects of antiestrogens. However, such lifelong exposure scenarios are problematic as they cannot discern whether the observed adverse impacts are due to organizational responses from larval exposure or the activational responses of adults that have been continuously exposed to estrogenic EDCs. This underscores the novelty of the present study that relied on early-life exposures followed by depuration until reaching sexual maturity.

The alternative explanation is that the EDCs used in these experiments do induce organizational effects, but their severity is dependent on the duration of exposure and subsequent depuration. As described in Chapter 3, compensation was observed in the gene expression and growth of minnow larvae following *in situ* exposure to an

agricultural runoff event at the Elkhorn River Research Station. In Chapter 4, adult fish showed a nearly full recovery from their early-life exposure to agriculturals except for the slight induction of an ovipositor in males exposed to both river water and sediments. Male fathead minnows also demonstrated compensation in reproductive performance following early-life feminization by fulvestrant (Chapter 5). Taken together, these observations support this alternative explanation indicating that, at a certain level of their biology, exposed organisms are capable of responding to the induction of organizational effects.

If the latter explanation is true, physiological compensation is an important caveat within the paradigm of activational and organizational responses. Fish are recognized to possess considerable physiological plasticity at early life stages, allowing them to compensate for early life stress caused by dynamic changes of the physicochemical parameters in their natural environments (Ali et al. 2003; Pittman et al. 2013). While it has been speculated that physiological compensation of larval fish is regulated by endocrine factors (Ali et al. 2003; Won and Borski 2013), relatively little work has considered this in the context of exposure to anthropogenic EDCs.

Future Directions

Relative to my broader goal of understanding the role of early-life xenobiotic exposure in adult health outcomes, it is evident that early life exposure to xenobiotic compounds followed by depuration under ideal conditions can produce subtle but not

adverse effects as adults. However, these findings also raise important questions for subsequent investigations relative to the role of early-life exposures in adult health outcomes. As an avenue of future research, there remain the questions of i) what are the biological mechanisms that elicit organizational effects on sexual development following early-life exposure to EDCs, ii) what are the limits of compensation in response to both natural and anthropogenic stressors during early development, and iii) how do the results of the present study translate to other vertebrate models.

Regarding the mechanism that initiates an organizational effect, there is a potential role of epigenetic modifications in the induction of persistent effects on sexual development following exposure to EDCs. Recent literature has emphasized the importance of epigenetic modification as a mechanism for the induction of persistent changes in the regulation of gene expression and their associated physiological functions (Vandeghehuchte and Janssen 2011; Pittman et al. 2013). However, the epigenome of the fathead minnow has yet to be characterized and only a single study has sought to identify important milestones for epigenetic programming in this environmental sentinel species (Wood et al. 2016). Does epigenetic programming of the gonad occur at the same time in both sexes, or are there differences in timing that play a role the observed differential susceptibility to organizational impacts from early-life exposure? If there is a role of epigenetic programming, such as the methylation of promotor sequences involved in sexual differentiation, there is potential for the identification of epigenetic biomarkers to predict the induction of organizational outcomes.

Another avenue of research would be the characterization of the limits of compensation following early-life exposures to better understand recovery mechanisms in vertebrate models. The depuration conditions in the previously described experiments were artificial, as the natural environment of the Elkhorn River and other aquatic systems are impacted by multiple sources of pollution that prevent the opportunity for aquatic organism to experience a true depuration period. Furthermore, the role of sediments in the mobilization of agrichemicals emphasizes that exposures likely persist beyond the initial runoff event, albeit limited to lower concentrations released over time or the increased dissociation during high-flow events (Kolok et al. 2014). Is the capacity for compensation for early-life exposures limited by the duration of exposure, the concentration of the compound or some combination thereof? Can the environmental conditions of the depuration period lead to overcompensation for certain early-life exposures? The answers to these questions will be important to understanding the impacts of episodic exposures to agricultural runoff, as well as developing a stronger understanding of often overlooked aspect of developmental toxicology.

A final direction for future research is the comparison of the results obtained from the fathead minnow with those obtained from other sentinel species. The results of Chapters 3 and 4 underscore the role of the aqueous and sediment matrices in exposure to endocrine-active agrichemical mixtures. Although the fathead minnow proved to be useful for identifying these matrix specific effects, it may not be the most appropriate sentinel species for studying the interactions of the environment and developmental toxicity in a river system like the Elkhorn River. One example of matching the sentinel

organism for the environment in questions would be the use of channel catfish (*Ictalurus punctatus*) that are abundant in Midwestern streams and have considerable economic value to the region (Eder et al. 2016), as well as naturally interacting with both surface water and sediments. The relatively longer lifespan of the channel catfish (Chapman 1992) versus that of the fathead minnow would present unique experimental challenges under laboratory conditions akin to those of the present study. However, their lifespan serves as a double-edged sword as it would be of tremendous value to wildlife epidemiology studies investigating the effects repeated seasonal exposures and depuration to agrichemicals in the natural populations.

Conclusion

Collectively, the findings in this dissertation provide evidence that early-life exposure to EDCs, including agrichemical runoff and pharmacological agents, are associated with organizational but not adverse outcomes in fathead minnows. This demonstrates that early-life exposure to xenobiotic compounds can affect adult outcomes; however, it also raises additional questions about the physiological mechanisms that allow for compensation and recovery from organizational responses. The interplay between the ontogenesis of adult dysfunction and early-life environments presents challenging yet intriguing avenues for future investigations.

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APPENDECIES

Supplemental Table 1

Pesticide compounds (include CAS# and molecular weight) measured in POCIS and sediment samples together with instrumental parameters, instrument detection limits (average IDL=20 pg) estimated from repeated injections of a 250 pg·uL⁻¹ calibration standard. Sediment method detection limits (MDL=t_{n-1}s) were estimated using the standard deviation of 8 replicate analyses of 5 gram of clean matrix fortified at 4 ng·g⁻¹. Recoveries = 100 × (measured/fortified). "NM" indicates not measured.

Compound	CAS#	Molecular Weight (g·mol ⁻¹)	Quantitation Ion (m·z ⁻¹)	Retention Time (min)	IDL (pg)	MDL Sediment (ng·g ⁻¹)	Recovery (%)
Acetochlor	34256-82-1	269.767	146	11.09	8.2	0.3	57.2
Alachlor	15972-60-8	269.767	160	11.47	6.6	1.6	34.5
Atrazine	1912-24-9	215.68	200	8.42	16.5	0.3	99.9
Butylate	2008-41-5	217.37	146	5.14	25.5	2.8	62.6
Chlorthalonil	1897-45-6	265.91	266	9.40	5.9	0.9	51.1
Cyanazine	21725-46-2	240.69	212	12.51	12.8	1.0	68.0
Deethylatrazine (DEA)	6190-65-4	187.63	172	7.17	29.3	2.3	114
Deisopropylatrazine (DIA)	1007-28-9	173.60	158	7.00	26.7	1.4	81.3
Dimethenamid	87674-68-8	275.79	154	10.84	2.9	3.2	103
EPTC	759-94-4	189.32	128	4.59	15.9	4.8	44.2
Metolachlor	51218-45-2	282.80	162	13.09	22.0	0.3	64.8

Metribuzin	21087-64-9	214.29	198	10.45	9.2	7.2	105
Norflurazon	27314-13-2	303.67	303	21.51	9.6	NM	NM
Supplemental Table 1 (continued)							
Compound	CAS#	Molecular Weight (g·mol ⁻¹)	Quantitation Ion (m·z ⁻¹)	Retention Time (min)	IDL (pg)	MDL Sediment (ng·g ⁻¹)	Recovery (%)
Pendamethalin	40487-42-1	281.31	252	14.68	28.5	3.1	128
Permethrin	52645-53-1	391.29	183	30.97	80.6	4.3	115
Prometon	1610-18-0	225.29	210	8.34	8.4	2.1	83.0
Propachlor	1918-16-7	211.69	120	6.71	91.1	0.6	70.4
Propazine	139-40-2	229.71	214	8.57	7.7	0.1	35.1
Simazine	122-34-9	201.66	201	8.20	3.9	0.7	120
Telfluthrin	79538-32-2	418.74	177	10.07	5.4	0.3	67.6
Trifluralin	40487-42-1	303.67	306	7.67	5.8	0.3	98.2

Supplemental Table 2

Primers used for real-time polymerase chain reaction analysis with respective annealing temperature and NCBI ascension number for each gene.

Gene	Forward sequence 5' - 3'	Reverse sequence 5' - 3'	Temp (°C)	Ascension Number
<i>ar^a</i>	GTTTCCGTAACCTG CATGTGG	CGCGCATTAGCGTTCT TGTA	60.9	AY727529
<i>cyp19a^b</i>	GCGGCTCCAGATAC TC	ACTCTCCAGAATGTTT AACC	55.0	AJ277867
<i>era^a</i>	AGTGAGCAGTCAA GCCGTGTT	GGTCAGGTGGCATGC ATAAAG	63.0	AY727528
<i>dmrt1^c</i>	AGGTCGTGGGTGAT GTGAAT	GGCCACTGCAGAGCT TAGAG	65.0	DT303249.1
<i>hsd17b^d</i>	ACAGCCAGCCGTAG AC	TCCTAAAGCCAGTGA TGAC	62.6	DT161033
<i>igf1^e</i>	GGCAAAACTCCAC GATCCCTA	ATGTCCAGATATAGGT TTCTTTGCTG	61.4	AY533140
<i>rpl8^a</i>	GCCCATGTCAAGCA CAGAAAA	ACGGAAAACACCTT AGCCAG	59.2	AY919670

^a Kolok et al. 2007, ^b Wood et al. 2015, ^c Leet et al. 2013, ^d Filby et al. 2006, ^e Beggel et al. 2011

Supplemental Methods for Chapter 4: Analytical Chemistry Methods for Water and Sediment

Water samples were extracted by solid phase extraction (SPE) for dissolved pesticide analysis. 100 mL raw water was filtered through 0.45- μ m glass microfiber filters and spiked with 2,000 ng surrogate compounds (d10-phenanthrene, terbutylazine, and butachlor) and 5,000 ng internal standards (13C3-atrazine, 13C3-deethylatrazine (13C3-DEA), and 13C3-deisopropylatrazine (13C3-DIA)). The mixture was directed under vacuum to Sep-Pak tC18 cartridge (Waters Corporation, MA, USA) preconditioned with successive 5 mL of ethyl acetate, methanol, and Nanopure water. Pesticides were then eluted from the cartridge using 4 mL ethyl acetate and residual water was removed by adding anhydrous sodium sulfate. This was concentrated to 300 μ L under nitrogen and transferred to a 400- μ L autosampler vial for gas chromatography mass spectrometry (GC/MS) analysis.

Sediment samples were extracted using MASE techniques with a MARS XPress (CEM Corporation, Matthews, NC) microwave system following previously described methods (Cassada et al. 1994; Smalling et al. 2008; Zhang et al 2015). Five grams (wet weight) of thawed sediment was weighed into a 10 mL TeflonTM microwave tube, mixed with 6 mL of acetonitrile and spiked with 400 ng of surrogate compounds. Samples were mixed by vortexing, and then microwaved at 800 W temperature ramped to 90 °C over 10 minutes and held at 90 °C for 5 min. After cooling to room temperature and allowing particles to settle, the acetonitrile was transferred to glass evaporation

tubes (RapidVap N2, Labconco, Kansas City, KS). An additional 10 mL of acetonitrile was added to the sediment to effect quantitative transfer and mixed by vortexing for 30 s. After settling, the second portion of acetonitrile combined in the evaporation tube and concentrated under nitrogen at 45°C until ~1-2 mL of extract remained. The concentrated extract was transferred to a glass culture tube, spiked with 1000 ng labelled internal standard, any residual water removed by pipetting, and then dried with anhydrous sodium sulfate. The extracted sample was then transferred to a second culture tube using ethyl acetate, evaporated to 100 µL, and then transferred to a 2 mL autosampler vial fitted with a 300 µL silanized glass insert using ~200 µL of ethyl acetate. Method detection limits (MDL) were determined by extraction of 8 replicates of sand spiked at 4 ng·g⁻¹ (Supplemental Table S1).

All water and sediment extracts were analyzed on an Agilent 5973 GC/MS outfitted with a Leap CombiPAL autosampler with split-less injection using a Restek (Bellefonte, PA) Rtx-1, 30m x 0.25 mm ID and 0.25 µm film thickness capillary column. Oven temperature was programmed to run at 80°C for 0.75 min, ramp to 170°C at 40°C·min⁻¹, ramp to 236°C at 2.5°C·min⁻¹, ramp to 275°C at 40°C·min⁻¹ and hold for 9.62 min. The injection port temperature was 250°C and the transfer line interface temperature was 280°C. Retention times, quantifying ions, and instrument detection limits determined from repeated analysis of the lowest standard are included in Supplemental Table S1. ¹³C₃-atrazine was used as the internal standard for all compounds except for DEA and DIA which used their respective labelled analogues.

Supplemental Table 3

Pesticide compounds (include CAS# and molecular weight) measured in POCIS and sediment samples together with instrumental parameters, instrument detection limits (average IDL=20 pg) estimated from repeated injections of a 250 pg·uL⁻¹ calibration standard. Sediment method detection limits (MDL=t_{n-1s}) were estimated using the standard deviation of 8 replicate analyses of 5 grams of clean matrix fortified at 4 ng·g⁻¹.¹ Method detection limits in water were estimated by replicate analysis of 8 100 mL aliquots of reagent water fortified at 0.5 µg L⁻¹.

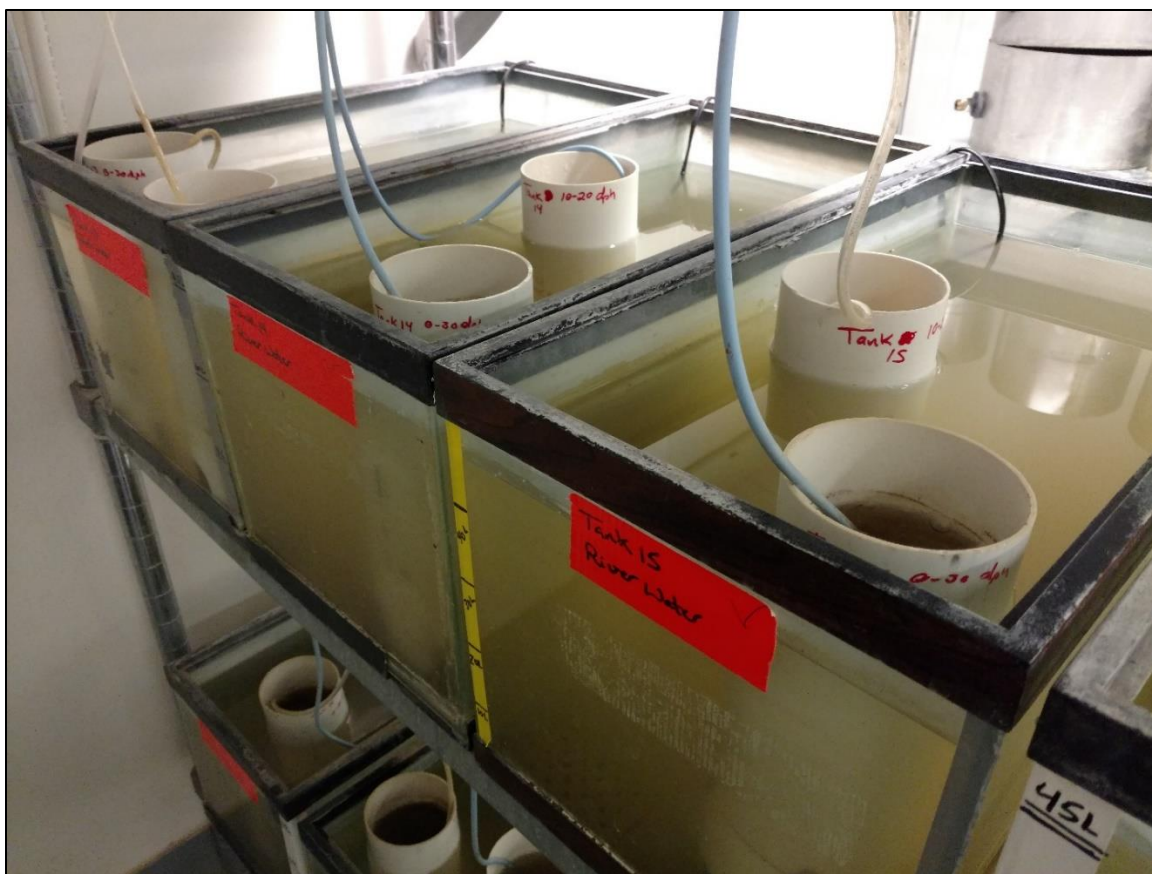
Compound	CAS#	Molecular Weight (g·mol ⁻¹)	Quantitation Ion (m·z ⁻¹)	Retention Time (min)	IDL (pg)	MDL Sediment (ng·g ⁻¹)	MDL Water (µg·L ⁻¹)
Acetochlor	34256-82-1	269.767	146	11.09	8.2	0.3	0.03
Alachlor	15972-60-8	269.767	160	11.47	6.6	1.6	0.03
Atrazine	1912-24-9	215.68	200	8.42	16.5	0.3	0.04
Butylate	2008-41-5	217.37	146	5.14	25.5	2.8	0.08
Chlorthalonil	1897-45-6	265.91	266	9.40	5.9	0.9	0.08
Cyanazine	21725-46-2	240.69	212	12.51	12.8	1.0	0.10
Deethylatrazine (DEA)	6190-65-4	187.63	172	7.17	29.3	2.3	0.02
Deisopropylatrazine (DIA)	1007-28-9	173.60	158	7.00	26.7	1.4	0.13
Dimethenamid	87674-68-8	275.79	154	10.84	2.9	3.2	0.04
EPTC	759-94-4	189.32	128	4.59	15.9	4.8	0.09
Metolachlor	51218-45-2	282.80	162	13.09	22.0	0.3	0.04

Metribuzin	21087-64-9	214.29	198	10.45	9.2	7.2	0.02
Norflurazon	27314-13-2	303.67	303	21.51	9.6	NM	0.08
Pendamethalin	40487-42-1	281.31	252	14.68	28.5	3.1	0.02
Compound							
Continued from previous page	CAS#	Molecular Weight (g·mol ⁻¹)	Quantitation Ion (m·z ⁻¹)	Retention Time (min)	IDL (pg)	MDL Sediment (ng·g ⁻¹)	MDL Water (µg·L ⁻¹)
Permethrin	52645-53-1	391.29	183	30.97	80.6	4.3	0.06
Prometon	1610-18-0	225.29	210	8.34	8.4	2.1	0.04
Propachlor	1918-16-7	211.69	120	6.71	91.1	0.6	0.07
Propazine	139-40-2	229.71	214	8.57	7.7	0.1	0.04
Simazine	122-34-9	201.66	201	8.20	3.9	0.7	0.03
Telfluthrin	79538-32-2	418.74	177	10.07	5.4	0.3	0.08
Trifluralin	40487-42-1	303.67	306	7.67	5.8	0.3	0.03

Chapter 4 Larval Exposure Setup

Supplemental Figure S1 shows the exposure aquaria setup for the 30-d larval exposure to their respective treatment. Due to the small size of fathead minnow, larvae were maintained within secondary exposure vessels made from a 10.5 x 25 cm PVC cylinder that sits on top of a 10.5 x 10 cm PVC joint with holes drilled into the bottom to allow for free flow of water around the vessel. The bottom of this exposure vessel was lined with 86 μ m steel mesh which has been shown to prevent the loss of fathead minnow larvae while allowing the free exchange of water and fine sediments under field conditions (Ali et al. 2016; Ali et al. 2017). The final volume of the larvae occupied space within these vessels was 1.70 L which could freely exchange water with the outside 45-L aquaria. This prevented both the loss of larval to the external aquaria and excessive agitation during water and sediment exchanges.

At the start of the exposure, 100 newly hatched larvae were randomly distributed into 2 L glass beakers containing 400 mL of filtered water. To prevent excessive stress to the larvae, water from their assigned treatment group was gradually added over a 5-h period to reach a final volume of 2 L before transferring them into their exposure vessels. Larvae remained in the same exposure vessel over the course of the 30-d larval exposure.

**Supplemental Figure 1**

Exposure aquaria for minnow larvae over the course of the 30-d period. Photograph shows river water treated aquaria with PVC larval exposure vessels used to prevent the loss of larvae.

Supplemental Table 4

Primers used for real-time polymerase chain reaction analysis with respective annealing temperature and NCBI ascension number for each gene.

Gene	Forward sequence	Reverse sequence	Temp (°C)	R ²	Efficiency (%)
<i>ar^a</i>	GTTTCCGTAACC TGCATGTGG	CGCGCATTAGCGT TCTTGTA	60.9	0.982	90.0
<i>era^a</i>	AGTGAGCAGTCA AGCCGTGTT	GGTCAGGTGGCAT GCATAAAG	63.0	0.959	108.9
<i>erβ^b</i>	ACAGTGTTTGAT GAGCTACG	GGAGTCCACCCAC CATATCGTGAT	61.1	0.993	91.6
<i>gr^b</i>	ACAGTGTTTGAT GAGCTACG	GGAGTCCACCCAC CATATCGTGAT	55.5	0.996	91.2
<i>igf1^c</i>	GGCAAAACTCC ACGATCCCTA	ATGTCCAGATATA GGTTTCTTTGCTG	61.4	0.986	112.8
<i>rpl8^a</i>	GCCCATGTCAAG CACAGAAAA	ACGGAAAACCAC CTTAGCCAG	59.2	0.997	79.7

^a Kolok et al. 2007

^b Filby et al. 2007

^c Beggel et al. 2011

Supplemental Table 5

. Physicochemical water quality parameters measured over the course of the 30-day larvae exposure for the filtered water (FW), filtered water with sediment (FWS), river water (RW) and river water with sediment (RWS) treatment groups. Values presented as mean (\pm standard deviation; n=120 per treatment group) of daily measurements from the four replicate aquaria within each treatment group.

Parameter	FW	FWS	RW	RWS
pH	8.3 (0.2)	8.3 (0.1)	8.6 (0.1)	8.5 (0.1)
Dissolved oxygen (%)	72.7 (8.4)	75.0 (10.7)	77.8 (7.1)	74.3 (5.8)
Dissolved oxygen (mg·L ⁻¹)	6.1 (0.8)	6.4 (0.8)	6.5 (0.6)	6.3 (0.8)
Conductivity	617.8 (42.3)	619.8 (35.1)	684.4 (51.1)	677.0 (61.0)
Total dissolved solids (mg·L ⁻¹)	407.2 (25.2)	408.4 (16.9)	455.2 (67.5)	464.2 (60.9)
Alkalinity (mg·L ⁻¹)	105.8 (8.4)	119.0 (6.3)	258.0 (11.9)	262.5 (13.5)

Supplemental Table 6

Two-way ANOVA results for all biological endpoints measured in 20- and 30-day post hatch minnow larvae. Where a significant effect was detected in the overall two-way ANOVA model specific effects are reported. Sample size per treatment group presented as n.

20 dph Larvae	n	Model (d.f.=3)	Water (W) effect (d.f.=1)	Sediment (S) effect (d.f.=1)	Interaction (W*S) (d.f.=1)
Body mass	20	F=3.528; p=0.0188	F=1.601; p=0.2097	F=1.307; p=0.2566	F=7.677; p=0.0070
Body length	20	F=2.968; p=0.0372	F=2.996; p=0.0876	F=1.236; p=0.2698	F=4.672; p=0.0338
Condition factor	20	F=1.349; p=0.2650			
ar/rpl8	7-10	F=6.985; p=0.0010	F=19.11; p=0.0001	F=0.757; p=0.3907	F=1.603; p=0.2146
era/rpl8	7-10	F=0.676; p=0.5744			
erβ/rpl8	8-10	F=1.086; p=0.3689			
gr/rpl8	8-10	F=2.285; p=0.0983			
igf1/rpl8	8-10	F=1.866; p=0.1552			
30 dph Larvae	n	Model (d.f.=3)	Water (W) effect (d.f.=1)	Sediment (S) effect (d.f.=1)	Interaction (W*S) (d.f.=1)
Body mass	20	F=6.040; p=0.0006	F=0.150; p=0.6993	F=5.798; p=0.0172	F=11.94; p=0.0007
Body length	20	F=3.248; p=0.0235	F=0.371; p=0.5436	F=1.925; p=0.1673	F=7.332; p=0.0075
Condition factor	20	F=7.020; p=0.0002	F=5.883; p=0.0164	F=12.51; p=0.0005	F=2.730; p=0.1005
ar/rpl8	8-10	F=4.383; p=0.0113	F=12.29; p=0.0015	F=0.246; p=0.6236	F=0.096; p=0.7587
era/rpl8	8-10	F=2.975; p=0.0457	F=2.704; p=0.1099	F=4.907; p=0.0342	F=1.873; p=0.1807
erβ/rpl8	7-10	F=1.726; p=0.1822			

Continued on next page.

gr/rpl8	7-10	F=2.098; p=0.1200
igf1/rpl8	7-10	F=1.488; p=0.2371
Survival ^a	4	F=3.120; p=0.0647

Statistical significance determined at $\alpha=0.05$.

^a Sample size indicates the number of replicate aquaria

Supplemental Table 7

Two-way ANOVA results for all biological endpoints measured in sexually mature female and male fathead minnows (165 days post hatch). Where a significant effect was detected in the overall two-way ANOVA model specific effects are reported. Sample size per treatment group presented as n.

Adult Females	n	Model (d.f.=3)	Water (W) effect (d.f =1)	Sediment (S) effect (d.f =1)	Interaction (W*S) (d.f =1)
Body mass	29-37	F=4.138; p=0.0074	F=0.026; p=0.8759	F=4.998, p=0.0268	F=6.045; p=0.0150
Body length	29-37	F=11.62; p<0.0001	F=0.093; p=0.7612	F=22.39; p<0.0001	F=10.59; p=0.0014
Condition factor	29-37	F=1.737; p=0.1617			
GSI	29-37	F=1.626; p=0.1868			
Relative ovipositor length	29-37	F=1.796; p=0.1504			
Adult Males	n	Model (d.f.=3)	Water (W) effect (d.f =1)	Sediment (S) effect (d.f =1)	Interaction (W*S) (d.f =1)
Body mass	29-34	F=5.354; p=0.0016	F=0.160; p=0.6899	F=0.000; p=0.9964	F=15.58; p=0.0001
Body length	29-34	F=7.936; p<0.0001	F=0.777; p=0.3794	F=0.128; p=0.7212	F=18.57; p<0.0001
Condition factor	29-34	F=4.085; p=0.0081	F=4.880; p=0.0288	F=1.644; p=0.2019	F=1.336; p=0.2497
Tubercle count	29-34	F=1.738; p=0.1620			
GSI	29-34	F=1.288; p=0.2823			
Relative ovipositor Length	29-34	F=5.090; p=0.0022	F=3.009; p=0.0850	5.964; p=0.0158	F=6.747; p=0.0104

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Breeding Assay	n	Model (d.f.=3)	Water (W) effect (d.f =1)	Sediment (S) effect (d.f =1)	Interaction (W*S) (d.f =1)
Final sex ratio ^a	4	F=0.913; p=0.4725			
Cumulative egg production	6	F=1.301; p=0.3015			
Number of clutches	6	F=0.733; p=0.5445			
Fecundity	6	F=1.670; p=0.2054			
% Fertilized Success ^b	8-11	F=0.345; p=0.7930			

Statistical significance determined at $\alpha=0.05$.

^a Sample size indicates the number of replicate aquaria

^b number of fertilization events scored over the 21-d breeding assay

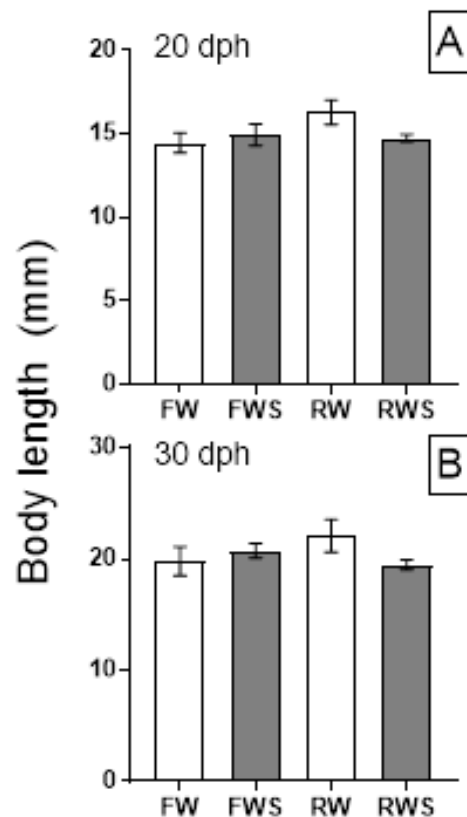
Supplemental Table 8

Results from the 21-day reproductive performance assay of sexually mature minnows with history of exposure to water, sediment or the water-sediment mixture. All values reported as the mean (\pm SEM) from of 6 breeding pairs per treatment group.

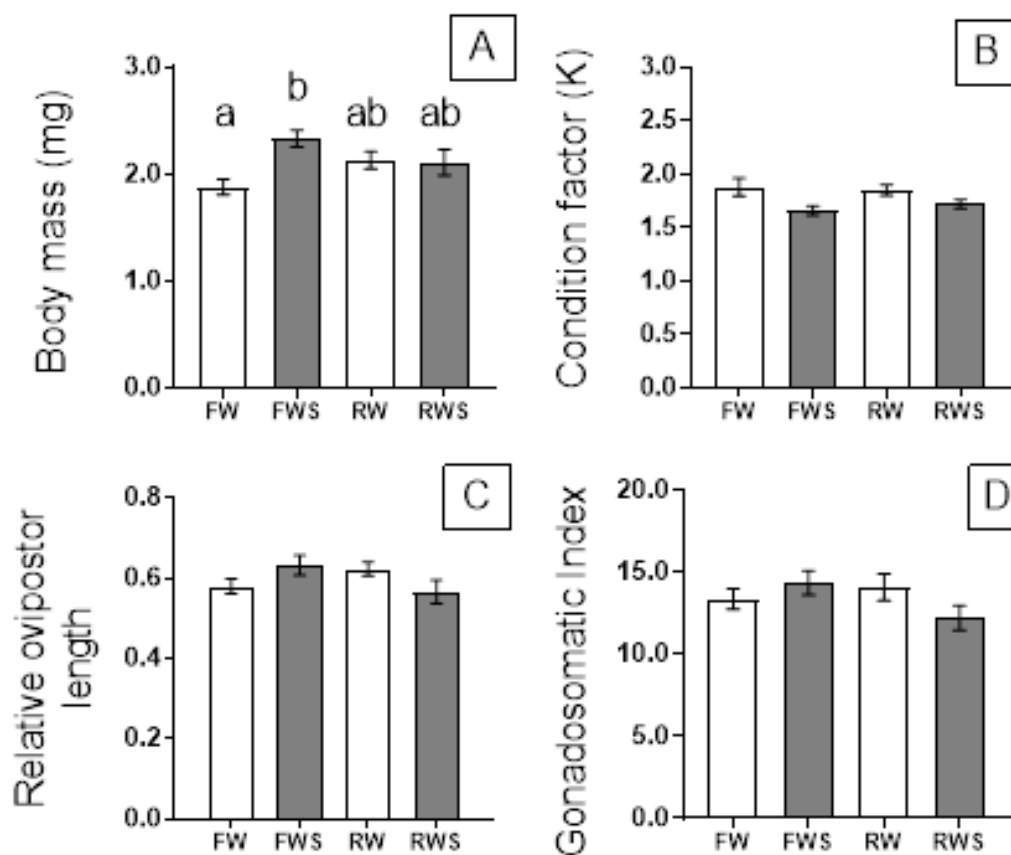
	FW	FWS	RW	RWS
Cumulative egg production ^a	1567 \pm 277	2099 \pm 418	1888 \pm 406	2571 \pm 360
Number of clutches ^a	5.8 \pm 0.8	5.5 \pm 0.9	6.5 \pm 1.0	7.5 \pm 1.3
Number of eggs/female/clutch, Fecundity	168.5 \pm 26.1	195.0 \pm 26.8	145.7 \pm 18.8	204.0 \pm 26.9
% Fertilized Success ^b	95.8 \pm 1.6	97.0 \pm 1.3	95.9 \pm 1.3	97.9 \pm 1.5

^a n=6

^b n=8-11

**Supplemental Figure 2**

Body length of fathead minnow larvae at 20- and 30-days post hatch. Bars represent the mean (\pm standard error) of larvae from the filtered water (FW), filtered water with sediment (FWS), river water (RW) and river water with sediment (RWS) treatment groups (n= 20 per treatment group).



Supplemental Figure 3

Morphometrics of sexually mature female fathead minnows with prior larval exposure to either filtered water (FW), filtered water with sediment (FWS), river water (RW) and river water with sediment (RWS) treatment groups. Bars represent the mean (\pm standard error, $n=24-37$ fish per treatment group) of each treatment group. Significant differences detected by Tukey's test for differences due to an interaction effect are denoted by lower case letters ($\alpha=0.05$).