THE IMPACT OF SEDIMENTS AND ENDOCRINE DISRUPTING COMPOUNDS ON FATHEAD MINNOWS

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Little is known about the impacts of sediment on the bioavailability of endocrine disrupting compounds to aquatic organisms. The goal of this thesis is to illuminate these impacts on the gene expression of fathead minnows (*Pimephales promelas*). To accomplish this goal, two studies were conducted. In the first, fish were exposed in the field to water from the Elkhorn River with varying levels of sediment during high and low discharge events. Significant reductions were observed in expression of both estrogen and androgen responsive genes, but only during a period of high discharge. Additionally, the response was dependent on the sediment load, with defeminization only occurring with whole sediment exposure and demasculinization occurring regardless of sediment profile. This study illustrates that direct contact with sediment can be necessary for some endocrine disrupting compounds to elicit their effects, thus emphasizing the importance of sediment in the bioavailability of agrichemicals.

The second study was designed to investigate the effects of a veterinary growth-promoting hormone used throughout the cattle industry in the region, 17-β-trenbolone. Preliminary evidence suggests that the 17-β-trenbolone metabolite composition and exposure duration may influence the biological response. Sediment was spiked with 17-

β-trenbolone and groups of fathead minnows were exposed to it for 5 or 10 days. Reduction in one estrogen responsive gene was observed only in fish exposed for days 0-5, which indicates that fish exposed to an endocrine disrupting compound for a short period of time may be able to recover, as evident by the lack of defeminization observed in the fish exposed for 10 days. Such a finding suggests that the effects of 17-β-trenbolone may be reversible.

Taken together, the studies described in this thesis validate the important role sediment has in the bioavailability of endocrine disrupting compounds, both agricultural pesticides and synthetic hormones.

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CHAPTER 1

GENERAL INTRODUCTION

Issues related to aquatic toxicology and its influence on animal physiology have received significant attention over the past several decades, due to the increased understanding of how many contaminants negatively affect animal endocrine systems (Colborn et al. 1993; Sumpter et al. 1998; Tyler et al. 1998). A large portion of the research in this field has explored the impact of wastewater treatment plants (Lye et al. 1997; Vajda et al. 2008; Barber et al. 2012) and pulp-papermills (Borton et al. 2009; Orrego et al. 2009). Another area of focus has been the influence of agricultural runoff and the contaminants that are carried with it, including chemicals from both row crop agricultural fields and beef cattle feedlots.

Recently, the influence of sediments on the bioavailability of these contaminants has been of particular focus. Several decades of research have shown that dissolved agrichemicals in watersheds elicit negative effects on the physiology of aquatic organisms (Colborn et al. 1993; Lye et al. 1997; Arcand-Hoy and Benson, 1998; Kolok et al. 2007; Sellin et al. 2009; Sellin et al. 2010, Knight et al. 2013; Ali and Kolok *In press*). Until recently, however only a relatively small amount of research has focused on the role of sediments in these systems (Schlenk et al. 2005; Duong et al. 2009), particularly in sediment-rich Midwestern watersheds. (Sellin et al. 2010; Zhang et al. *Submitted*).

AIM STATEMENT

The goal of this thesis is to further illuminate the impacts of sediment and endocrine disrupting compounds on fathead minnows, which will advance the understanding in this growing field. Evidence from background research and preliminary projects indicated that sediment not only has a role in aquatic toxicology, but also may be a driving force in eliciting observable endocrine disruption. This has led to the hypothesis that sediment characteristics, such as particle size and composition, as well as varying exposures to sediment-associated agrichemicals and their metabolites would cause differing effects on fathead minnows. To address this hypothesis, two projects were developed, with the aims of (1) determining the effects of sediments on the bioavailability of agrichemicals in the field and (2) evaluating the effects of duration of exposure to a sediment-associated endocrine disrupting steroid as its metabolite profile changes,

THESIS ORGANIZATION

This thesis is divided into 5 chapters. Chapter 2 of this thesis provides a literature review, investigating background work and the projects used to develop this thesis. Topics include water contamination, a review of endocrine disruption in aquatic organisms, background information on agrichemical impact on the Elkhorn River (Nebraska, USA), and issues related to the timing of exposure to endocrine disrupting compounds.

Chapter 3 describes a field study on the Elkhorn River, investigating the impact of sediment on the bioavailability of agrichemicals to aquatic organisms, in particular, the model organism *Pimephales promelas*, the fathead minnow. Two week long field exposures were conducted, one during a low discharge event and the other during a high discharge event caused by precipitation. This allowed for an evaluation of the impact of high discharge events, which are suspected to carry high loads of contaminated sediments. Additionally, a cascade system was used to separate sediments, creating a mesocosm with whole sediment another with only a fine fraction. These conditions allowed for the investigation of the impact of the profile to which aquatic organisms are exposed.

Chapter 4 presents an in house project studying the effects of exposure intervals to an endocrine disrupting steroid, 17-β-trenbolone, as its metabolite profile in the sediment and water column changes, also on fathead minnows. Fathead minnows were housed for 5 or 10 days in a tank with sediment that had been spiked with a small dose of 17-β-trenbolone. The purpose of this was to investigate the effect of a short-term exposures of this steroid and compare it to the effect of a longer exposure to an attenuating level of the compound and its metabolites. Finally, Chapter 5 is the general conclusions drawn from both projects, as well as acknowledgments and references.

CHAPTER 2

ENDOCRINE DISRUPTION

CONTAMINATION IN THE AQUATIC ENVIRONMENT

Throughout the United States, waterways have been found to commonly contain chemical compounds that are the result of the production, use, and disposal of numerous industrial, agricultural, medical, and household chemicals (Daughton and Ternes 1999; Kolpin et. al 2002; Glassmeyer et al. 2005; Benotti et al. 2009). Evidence suggests that the most common wastewater treatment practices do not completely eliminate an array of these common contaminants (Petrovic et al. 2003; Falconer et al 2006). For example, a comprehensive review (Liu et al. 2008) of the literature on the removal of common agrichemicals suggests that the efficiency of current practices range from virtually complete (99.9%) to a modest 10%. This is primarily due to the fact that common physical methods of treatment are relatively ineffective, coupled with the slow acceptance of the more efficient biological wastewater treatment processes (Chang et al. 2007). Because of this lack of complete removal, there has been a growing body of research over the past several decades on the fate and bioavailability of these compounds in the water.

Although the physical and chemical mechanisms behind sediment-associated contaminants have received considerable research (Wauchope 1978; Pereria and Rostad 1990; Chapman et al. 2005), little has been done on the biological effect of these agrichemicals that are associated with sediment. The focus has remained on the biological effect of contaminants primarily in the dissolved forms. However, Midwestern waters have suspended sediment concentrations far higher than waterways in the eastern and western portions of the country, with levels from double to ten times the amount in other regions (Lee and Glyson, 2013). These sediment levels increase following precipitation events, and the runoff from row crop and cattle feedlots contain high levels of pesticides and steroids used by these industries (Wauchope 1978; Krutz et al. 2005). In fact, runoff is considered the primary agricultural mechanism contributing to surface water contamination (Krutz et al. 2005). With this in mind, it is important that the bioavailability of sediment-associated contaminants be understood, so that the short and long term effects on aquatic species may be understood.

ENDOCRINE DISRUPTING COMPOUNDS

A nationwide sampling survey of 139 streams in the US found 82 common chemicals in most of these streams, 33 of which have been shown to disrupt animal endocrine systems (Kolpin et al. 2002). Research also shows that these compounds can disperse and persist in the environment for a significant amount of time, from days to years (US CDC 2003; US EPA 2006). There is a milleu of research on the effect these exogenous compounds can have on the endocrine systems of the organisms which are exposed to them for both acute and chronic time periods, from invertebrates (Zou 2005; Iguchi and Katsu 2008) to vertebrates (Kolok et al. 2007; Sellin et al. 2008; Sellin et al. 2009; Knight et al. 2013; Ali and Kolok *In press*; Zhang et al. *Submitted*), including humans (Colborn et al. 1993; Munger et al.1997). These compounds have been appropriately dubbed endocrine disrupting compounds, or EDCs, due to their adverse effects on hormone regulation.

EDCs are generally considered, “chemicals whose primary effect is on the endocrine system via effects on receptor mediated hormone action, hormone synthesis, or clearance" (Pickering and Sumpter 2003). In a comprehensive mini-review of the mode of action of EDCs, Tabb and Blumberg (2006) concluded that these chemicals have can elicit their effects via a wide range of molecular mechanisms, from mimicking endogenous ligands (Norris and Carr 2006; Li et al. 2013) to manipulating DNA methylation (Razin and Kantor 2005) and sensitizing hormone receptors (Jansen et al 2004). Point sources of EDCs include wastewater treatment plants and industrial wastewater, such as pulp-papermill effluent (Zhang and Zou 2008; Thorpe et al. 2009; Orrego et al. 2009; Van de Heuval 2010; Lee et al. 2013). However, there are non-point sources to consider as well, such as runoff from non-permeable surfaces in cities and suburban areas (Yang and Carlson 2003; Barnes et al. 2004) as well as runoff from rural agricultural fields. Additionally, research confirms that EDCs exist in a variety of chemicals used in agricultural practices, such as pesticide application on row crops, which can get swept up in surface water runoff following precipitation events and enter both lentic and lotic bodies of water that are used as sources of drinking water (Benotti et al. 2009).

FATHEAD MINNOWS AS A MODEL ORGANISM

The effects that EDCs have on living things often involve exposing a surrogate species to contaminants in laboratory or field situations. There have been several model fish organisms for the past several decades on aquatic toxicology studies that have proved useful as indictor species for ecotoxicology, the fathead minnow (*Pimephales promelas*) among them. Biomarkers of exposure include morphological to molecular changes, including changes in secondary sex characteristics, sex ratios, and hormone levels, and alteration of gene expression (Parrott and Blunt 2005; Orlando et al. 2004). In particular, fathead minnows exposed to agrichemical runoff have proven to experience alterations in secondary sex characteristics, fecundity, blood plasma protein levels, morphology, and survivorship (Ankley et al. 2002; Ankley et al. 2003; Martinović et al. 2008). Additionally, changes in gene expression associated with sex hormones are commonly observed (Hutchinson et al. 2006; Kolok et al. 2007; Sellin et al. 2008; Sellin et al. 2009; Knight et al. 2013; Ali and Kolok *In press*). Such changes in gene expression have been the endpoint of interest in the ATL and thus are the focus of this thesis.

SEDIMENTS IN MIDWESTERN WATERS

In intensely agricultural watersheds, agrichemicals are mobilized from the land surface during seasonally driven precipitation events (Thurman et al. 1991; Coupe et al., 1993; Hyer et al. 2001; Knight et al. 2013). Such seasonal pulses expose aquatic organisms to endocrine disrupting contaminants, with concentrations as high as 100 times concentrations detected during the post-pulse period (Knight et al. 2013). These pulse events lead to biological responses in organisms, as previous work has shown that female fathead minnows exposed to agrichemicals during a pulsatile runoff event experienced molecular defeminization while those exposed after the pulse did not (Sellin et al. 2009; Knight et al. 2013).

Waterways in agricultural areas often contain suspended solids up to tenfold higher than concentrations typically detected in other regions (Lee and Glysson 2013), and the presence of sediment can enhance the mobility of metals (Carter et al. 2006; Izquierdo et al. 2012), nutrients (Kronvang et al. 1997; Ouyang et al. 2012), and organic contaminants (Soares et al. 2008; Writer et al. 2011). Recent laboratory studies have demonstrated that sediment-associated pesticides and steroids are still bioavailable and have the potential to elicit endocrine disrupting effects (Sellin et al. 2010; Sangster et al. 2014; Jessick et al. 2014). Sellin et al. (2010) reported that female fathead minnows exposed to river sediment were defeminized, whereas those exposed to only river water were not. Sangster et al. (2014) revealed that trenbolone-contaminated sand and silty loam sediments induced significantly different biological responses in fish, indicating sediment properties might influence the bioavailability and toxicity of endocrine disrupting compounds.

The route by which organisms are exposed to sediment-bound compounds remains uncertain. Early studies demonstrated that ingestion of sediment could be the dominant route for exposure of deposit-feeders to hydrophobic compounds (Thormann et al. 1992; Lu et al. 2004). Jessick et al (2014) showed that fish exposed to steroid-containing sediment experienced significant defeminization without direct sediment contact, implicating ventilation of desorbed steroids as another exposure pathway. These laboratory studies demonstrated that sediment could either be directly involved in aquatic organism exposure to endocrine-disrupting compounds or a secondary source when compounds are released from sediment into the water column. However, the relative importance of direct exposure to sediment bound contaminants has not previously been evaluated in the field.

The objective of my first study was to evaluate the role of direct sediment exposure on the bioavailability of agrichemicals to aquatic organisms in the field. To achieve this objective, a mesocosm study was conducted on the Elkhorn River in Nebraska, located within an intensively agricultural watershed. This study featured an exposure system in which fish were exposed to either raw river water containing naturally occurring sediment or river water containing only the fine particle fraction of the sediment.

Previous work investigating the biological effects of Elkhorn River exposure led to my hypothesis that effects would only be observed during a pulsatile event, and that the effects would be more pronounced in fish exposed to complete sediment loads.

THE IMPORTANCE OF EXPOSURE DURATION

As stated previously, endocrine disrupting compounds include pesticides, industrial wastes, and veterinary pharmaceuticals (Kolpin et al. 2002). One such veterinary pharmaceutical is trenbolone acetate, a commonly used growth-promoting hormone in the cattle industry (Ankley et al. 2003). 17-β-trenbolone is one of the metabolites of the biotransformation of this steroid, which is excreted by cattle and enters aquatic systems (Schiffer et al. 2001). This is of particular concern in the Central United States, where approximately half of the land use is dedicated to grazing (Nickerson et al. 2011). Several studies (Soto et al. 2004; Orlando et al. 2004; Kolok et al. 2007) have confirmed that sediment from watersheds associated with cattle feedlots contain high levels of this steroid. Although it is not the most prevalent metabolite of trenbolone acetate, 17-β-trenbolone is considered relatively stable in the environment (Webster et al. 2012) and has the highest binding affinity to the androgen receptor (Schiffer et al. 2001; Ankley et al. 2003). Thus, it is important to understand the fate and transport of this compound in the environment and its biological effect in aquatic systems.

While Ankley et al. (2003) did determine that exposure to aqueous 17-β-trenbolone induced defeminization in fathead minnows, little is known about its interaction between sediment and its influence on defeminization. Given the hydrophobic nature of this compound, the dynamic equilibrium between the sediment and water column cannot be ignored. Empirical evidence (Sangter et al. 2014) shows that sediment does indeed influence whether or not a biological effect is observed. Fish exposed to sediment-associated 17-β-trenbolone showed reduced production of the egg-yolk precursor protein vitellogenin (*VTG*), while those exposed to an aqueous solution did not. Furthermore, preliminary unpublished data from our lab shows that even low doses of 17-β-trenbolone induce defeminization in just four days. However, fish exposed for a short period of time were able to slightly recover when removed from the contaminated sediment. This implies that the time period and duration to which the fish are exposed affects the degree of defeminization.

The objective of this second study was to determine the impact of exposure intervals to an endocrine disrupting steroid as its metabolite profile in the sediment and water column changes. To achieve this objective, fish were exposed to a controlled in-house sediment and water mixture for various time periods and analyzed for changes in gene expression of two estrogen-responsive genes, vitellogenin (*VTG*) and estrogen-receptor alpha (*ERα*).

CHAPTER 3

IMPACT OF SEDIMENT ON THE BIOAVAILABILITY OF AGRICHEMICALS TO ADULT FEMALE FATHEAD MINNOWS: A FIELD STUDY

INTRODUCTION

To date, more than a hundred pesticides have been identified that elicit adverse endocrine disrupting effects, including humans (McKinlay et al. 2008). For example, the herbicide atrazine, exhibits an estrogenic effect on steroid metabolism, and is also an androgen antagonist, although the specific mechanism is unknown (Cocco 2002; Cooper et al. 2000; Sanderson et al. 2000; Thibaut and Porte 2004). In addition to pesticides, growth-promoting compounds used in animal production contain various estrogens and androgens that are released into aquatic systems after land application of animal manure (Kolok and Sellin, 2008). These hormones and their metabolites can induce (de)feminization and (de)masculinization in fish (Jenkins et al. 2003; Filby et al. 2007) and cause intersex and biased sex ratio at environmentally relevant concentrations (Jenkins et al. 2003; Filby et al. 2007; Vajda et al. 2008).

In intensely agricultural watersheds, agrichemicals are mobilized from the land surface during seasonally driven precipitation events (Thurman et al., 1991; Coupe et al., 1993; Hyer et al., 2001; Knight et al. 2013). Such seasonal pulses expose aquatic organisms to endocrine disrupting contaminants, with concentrations as high as 100 times concentrations detected during the post-pulse period (Knight et al. 2013). These pulse events lead to biological responses in organisms, as previous work has shown that female fathead minnows exposed to agrichemicals during a pulsatile runoff event experienced molecular defeminization while those exposed after the pulse did not (Sellin et al., 2009; Knight et al. 2013).

Waterways in agricultural areas often contain suspended solids up to tenfold higher than concentrations typically detected in other regions (Lee and Glysson 2013), and the presence of sediment can enhance the mobility of metals (Carter et al., 2006; Izquierdo et al., 2012), nutrients (Kronvang et al., 1997; Ouyang et al., 2012), and organic contaminants (Soares et al., 2008; Writer et al., 2011). Recent laboratory studies have demonstrated that sediment-associated pesticides and steroids are still bioavailable and have the potential to elicit endocrine disrupting effects (Sellin et al., 2010; Sangster et al., 2014; Jessick et al., 2014). Sellin et al. (2010) reported that female fathead minnows exposed to river sediment were defeminized, whereas those exposed to only river water were not. Sangster et al (2014) revealed that trenbolone-contaminated sand and silty loam sediments induced significantly different biological responses in fish, indicating sediment properties might influence the bioavailability and toxicity of endocrine disrupting compounds.

The route by which organisms are exposed to sediment-bound compounds remains uncertain. Early evidence suggests that ingestion of sediment could be the dominant route for exposure of deposit-feeders to hydrophobic compounds (Thormann et al. 1992). Jessick et al (2014) showed that fish exposed to steroid-containing sediment experienced significant defeminization without direct sediment contact, implicating ventilation of desorbed steroids as another exposure pathway. These laboratory studies demonstrated that sediment could either be directly involved in aquatic organism exposure to endocrine-disrupting compounds or a secondary source when compounds are released from sediment into the water column. However, the relative importance of direct exposure to sediment bound contaminants has not previously been evaluated in the field.

The objective of this study was to evaluate the role of direct sediment exposure on the bioavailability of agrichemicals to aquatic organisms in the field. To achieve this objective, a mesocosm study was conducted on the Elkhorn River in Nebraska, located within an intensively agricultural watershed. This study featured an exposure system in which fish were exposed to either raw river water containing naturally occurring sediment or river water containing only the fine particle fraction of the sediment. Previous work investigating the biological effects of Elkhorn River exposure led to my hypothesis that effects would only be observed during a pulsatile event, and that the effects would be more pronounced in fish exposed to complete sediment loads.

MATERIALS AND METHODS

*Site Description***.** The field study was conducted at the Elkhorn River Research Station (ERRS). This field station is located approximately 10 km above the confluence of the Elkhorn and Platte Rivers on the western edge of Omaha, NE. Land use within the Elkhorn River Basin is mixed;-roughly half of the land area within the watershed is row crops field, with the remaining land in the western half primarily used to graze livestock (NDEQ, 2009). Confined beef cattle feedlots are common in the eastern portion of the watershed, whereas cow and calf grazing is common in the west. Historically, the river discharge is lowest in the winter and highest during the spring snowmelt and precipitation events (USGS, 2014). These episodic precipitation events carry large quantities of various agrichemicals, including steroid hormones and pesticides, which originate from the intensive agricultural practices throughout the Elkhorn River watershed (Kolok et. al. 2009; Sellin et. al. 2009).

*Atrazine as an Indicator of the Agrichemical Seasonal Pulse***.** For this study, rapid assessment atrazine test strips (Abraxis, Warminster, PA, USA) were used to indicate the presence of elevated agrichemical concentrations in the river. The test strips detect levels of atrazine greater than 3 ppb, which is the US EPA drinking water standard. In previous studies (Knight et al. 2013; Ali and Kolok *In press*), positive test strips have correlated with high concentrations of various agrichemicals, supporting the use of atrazine as a sentinel chemical that indicates the presence of a suite of other compounds. Testing began on April 30th and continued every 3-4 days until June 22nd. The first positive test strip occurred on May 12th, which was interpreted as the beginning of the spring pulse. The last positive strip was observed on June 10th. Subsequent tests on June 18th and 22nd were negative, indicating the end of the spring pulse.

*Test Organism***.** Sexually mature female fathead minnows (*Pimephales promelas*) from the University of Nebraska at Omaha Aquatic Toxicology Laboratory (ATL) colony were used for this study. Prior to experimentation, all fish were maintained in 30 L aerated aquaria with dechlorinated lab water, maintained at 25 ± 1^{o}C, and kept under a 16:8 light:dark photoperiod. Fish were fed TetraMin flake food (Aquatic EcoSystems, Apopka, FL, USA) twice daily and received a daily one-third static renewal water change. No males were used in this study, as previous work (Knight et al. 2013) showed no effect in males from exposure to water from the Elkhorn River during the spring pulse.

Fish Exposure System. The ERRS is designed to pump river water into mesocosms used for aquatic organism exposures and the photoperiod, water temperature and the biotic environment (i.e., planktonic community) are the same as that of the river. In each mesocosm, a 10-L stainless steel circular exposure tank was maintained within a 16.5-L insulation tank. The insulation tanks circulated river water to maintain ambient river water temperature in the exposure tanks.

Three mesocosms were designed into a cascade system (Figure 1). Water was pumped into the upper mesocosm, drained into the one beneath it, and finally drained into the lower mesocosm. Flow rates in the systems were maintained at approximately 500 mL/min. Due to the residence time of the water in the tanks and in accordance with Stoke's law, larger particles settled in the upper mesocosm, small silt particles in the middle mesocosm, and only fine particles were carried into the lower mesocosm. The

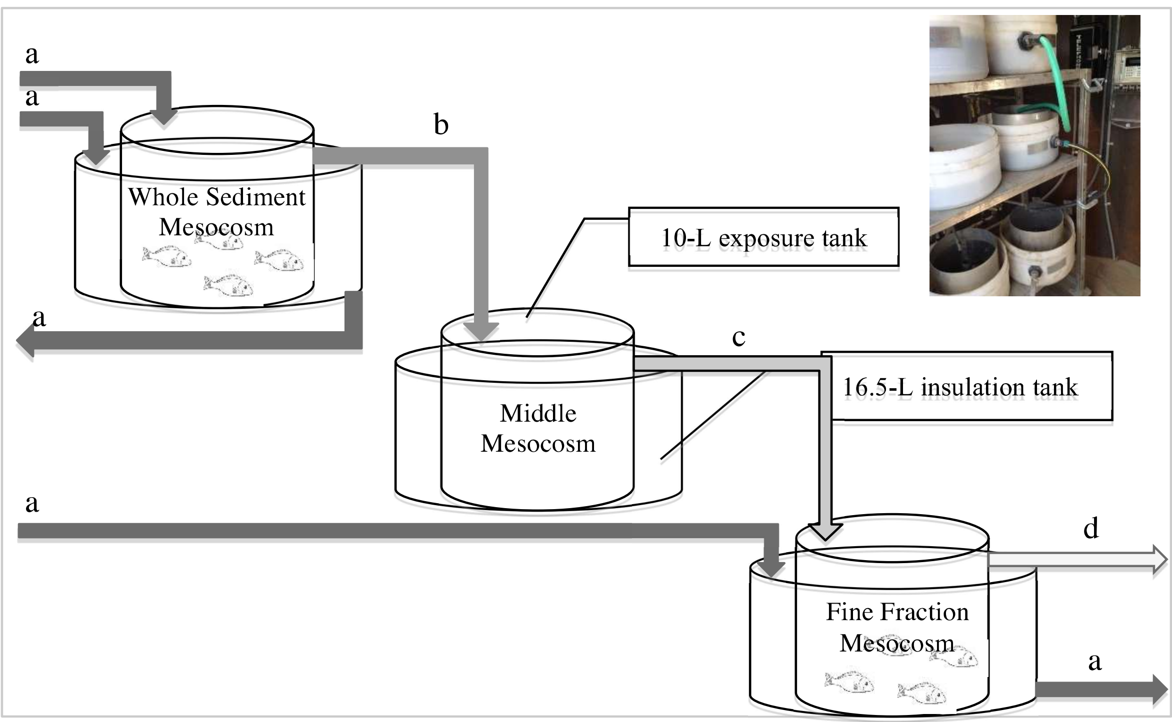

Figure 1. A schematic diagram of the fish exposure system (a) raw river water; (b) whole sediment mesocosm effluent carrying fine fraction and relatively bigger silt particles; (c) middle mesocosm effluent carrying only fine fraction; (d) fine fraction mesocosm effluent carrying extremely fine particles.

total amount of sediment present in the lower mesocosm was 10% of the whole sediment in the upper mesocosm. Particle size analysis confirmed these assumptions upon completion of the study. These sediment characteristics led to the designation of the upper mesocosm as "whole sediment" and the lower mesocosm as the "fine fraction".

Two 7-d fish exposure experiments were conducted during the spring pulse. For each week, twelve female fathead minnows were placed in both the whole sediment and fine fraction mesocosms for each of the 7-d exposures. No fish were deployed in the middle tank. Thus, there were two groups of organisms exposed to either river water containing whole sediment or the fine fraction during a low discharge week (week 1) and a high discharge week (week 2). These treatment groups were compared to a control group of twelve random females that remained at the ATL. All fish were weighed and mass recorded prior to placement in their respective group. During each 7-d period, fish in both the field and ATL were fed once daily with TetraMin flake food. In addition, the control fish in the ATL received a daily one-third static renewal water change.

At the end of each of the 7-d exposures, the field fish were taken back to the ATL in aerated coolers containing the water they were exposed to in the mesocosms. Once back at the laboratory, the fish from all three groups were lethally anaesthetized with 300 mg/L tricaine methanesulfonate (MS-222; Sigma Aldrich). Body mass was recorded, and liver and gonads were collected, weighed, and flash-frozen in liquid nitrogen. Tissues were stored at -80 ^{o}C until further analysis. Gonadosomatic index (GSI) and hepatosomatic index (HSI) were generated for each individual by dividing the mass of the tissues by the total body mass and multiplying by 100. There were minor mortalities in several of the groups for both weeks. Additionally, as it is oftentimes difficult to differentiate between a female and a subordinate male, some of the tanks had males that were not obvious until the end of the exposure. Thus, not every treatment group had 12 individuals.

Real-time PCR and Biological Analysis. Tissues used for quantitative real-time polymerase chain reaction (QPCR) utilized the SV Total RNA Isolation System (Promega, Sunnyvale, CA, USA) and the manufacturer's protocol to extract RNA. The RNA was resuspended and stored in nuclease-free water at -80°C until further analysis. The purity and concentration of the RNA was determined by Nanodrop (NanoDrop Technologies, Wilmington, DE, USA). Purity was assessed based on optical densities at 260 nm/230 nm. The samples were diluted to 10 ng/µL in preparation for cDNA synthesis. First-strand cDNA synthesis was performed with 0.75 µg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) in accordance to the manufacturer's recommendations. The QPCRs were performed using the iQ SYBR-Green Supermix (Bio-Rad) in accordance to the manufacturer's protocol. Two µL of diluted cDNA template was added to 300 nM forward and reverse primers in a 13 µL volume containing SYBR-Green Supermix, for a total volume of 15 µL. Two estrogen-responsive genes, *VTG* and *ERα*, and the androgen receptor, *AR*, were selected for analysis; primer sequences were obtained from Kolok et al. 2007. In addition, the housekeeping gene ribosomal protein L8 was used to normalize gene expression using the $2^{-\Delta C'T}$ method (Livak and Schmittgen 2001).

Biological Data Analysis. Morphometric and relative gene expression data were analyzed by ANOVA followed by Tukey post-hoc comparisons. If assumptions of ANOVA were violated, data were analyzed by Kruskal-Wallis nonparametric comparison followed by a Wilcoxon rank-sum test (as was the case for *ERα* in week 2).

Water Quality Sampling and Analyses. Water quality monitoring began on May 6th and continued until June 13th, several days after the last fish exposure was completed. Water temperature, pH (YSI 63, YSI Incorporated, Yellow Springs, OH, USA), dissolved oxygen (DO; YSI 55, YSI Incorporated, Yellow Springs, OH, USA), total suspended solids (TSS; 3150, InsiteIG, Slidell, LA, USA), and turbidity (WQ770-B, Global Water, Gold Water, CA, USA) were measured daily on site. Water and sediment samples were collected every 3 days as well as after precipitation events. All samples were shipped on ice to the Water Sciences Laboratory (WSL) at the University of Nebraska-Lincoln where water samples were analyzed for total pesticides and sediment samples were analyzed for pesticides and steroid hormones.

RESULTS

Biological Effects. No significant differences were detected among the treatment groups in body mass, HSI or GSI for both exposure periods. Results for gene expression analysis are presented in Figure 2. During week 1, there were no significant differences among the control and treatment groups in relative mRNA expression of *VTG*, $ER\alpha$, or *AR*. However, differences were observed in week 2. Both treatment groups experienced significant reduction in *AR* expression (ANOVA, $p < 0.05$, Tukey). However, only fathead minnows exposed in the whole sediment mesocosm had reduced expression of *VTG* and $ER\alpha$ (ANOVA, $p < 0.05$, Tukey/Wilcoxon) with respect to the controls, while those exposed to a fine fraction did not show expression reduction in these genes.

Agrichemical Occurrence and Concentration. Data on discharge (USGS Gaging Station) and pesticide concentrations, in both the dissolved and sediment-associated forms (Water Science Lab, Lincoln, NE), are presented in Figures 3 and 4, respectively. Specific

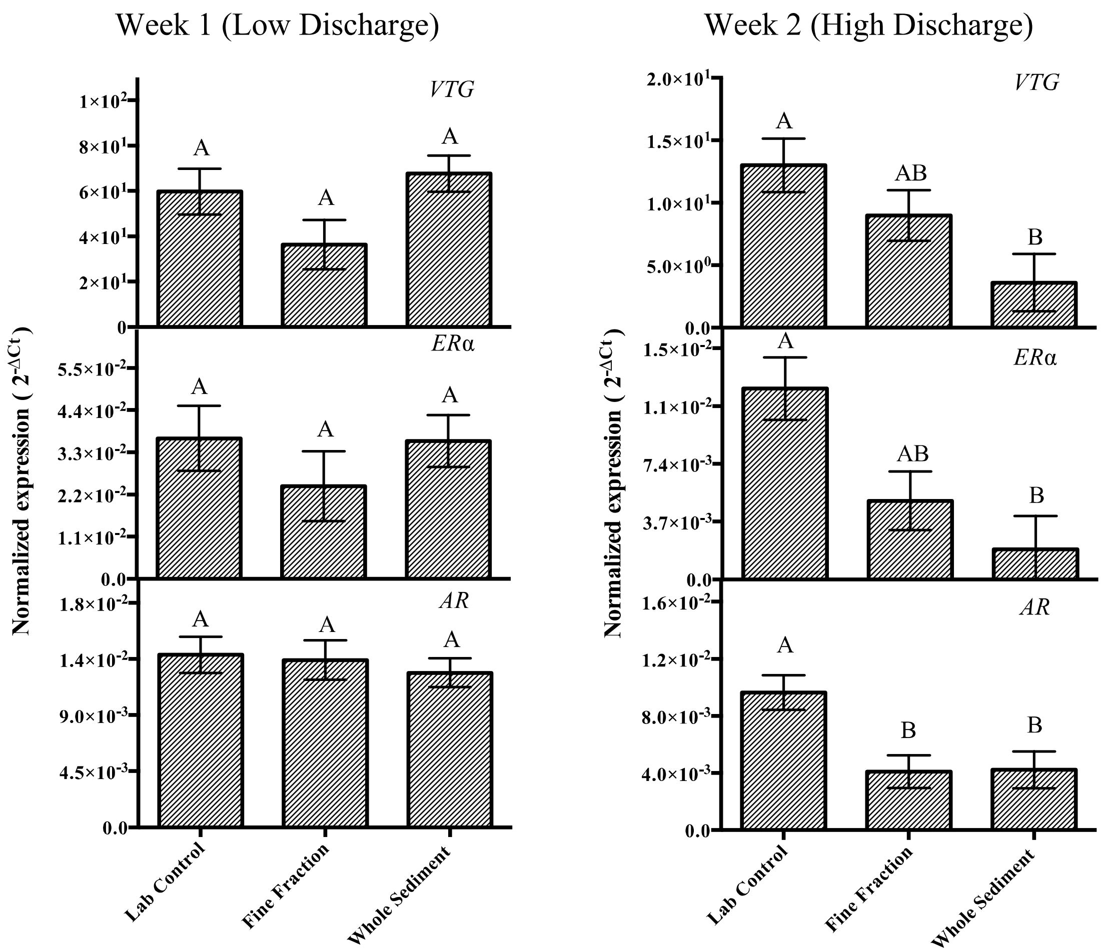

Figure 2. Effects on hepatic mRNA expression of three endocrine responsive genes in female fathead minnows in low discharge week and high discharge week. Significant differences denoted by capital letters as determined by one-way ANOVA or Wilcoxon test. Values represent mean (±SE), sample size of 6-11 fish per treatment group. *VTG* = vitellogenin, *ERα* = estrogen receptor alpha, *AR* = androgen receptor.

pesticide species and concentrations are presented in Zhang et al. (*In* review). During week 1, river discharge remained below 2000 ft^3/sec, while a peak of over 10,000 ft^3/sec occurred during week 2 after a precipitation event. Week 1 combined pesticide concentrations remained below 1.5 ppb and 46 ppb in the dissolved and sediment-associated phases, respectively. During week 2, these concentrations reached 14.76 ppb in the dissolved phase and 92.06 ppb in the sediment. No steroids were detected during week 1. 4-Androstenedione and 17-β-Estradiol were detected during week 2, at concentrations of <0.5 ppb and at 1.73 ppb, respectively.

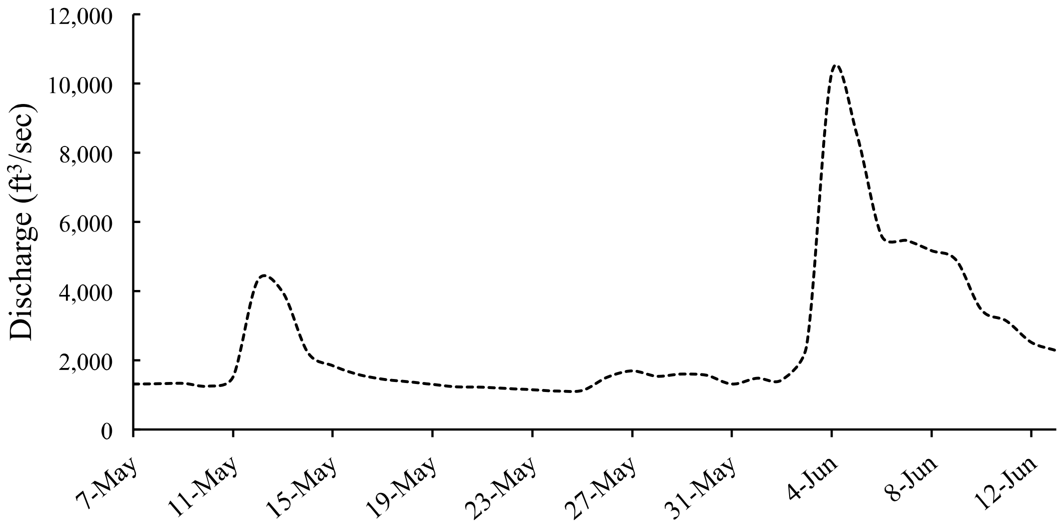

Figure 3. Elkhorn River discharge (a) taken from USGS Gaging Station (06800500) at Waterloo, NE. Boxes indicate the two 7-d fish exposures.

DISCUSSION

The Impact of River Discharge on Fathead Minnow Gene Expression. During week 1, there were no significant changes in *VTG*, *ER*α*,* or *AR* among the controls and the treatment groups. The most likely reason for this was the lack of precipitation events occurring during this time and the resulting lower concentrations of pesticides in both the dissolved and sediment-associated phases. In contrast to week 1, the week 2 exposure occurred during a major discharge event (Figure 3). A statistically significant down-regulation of both estrogenic (*VTG* and *ER*α) and the androgenic (*AR*) gene was observed in week 2 due to exposure to a complex mixture of various endocrine disrupting compounds. It is interesting that defeminization and demasculinization could occur on the same group of subjects at the same time. To our best knowledge, this is the first study

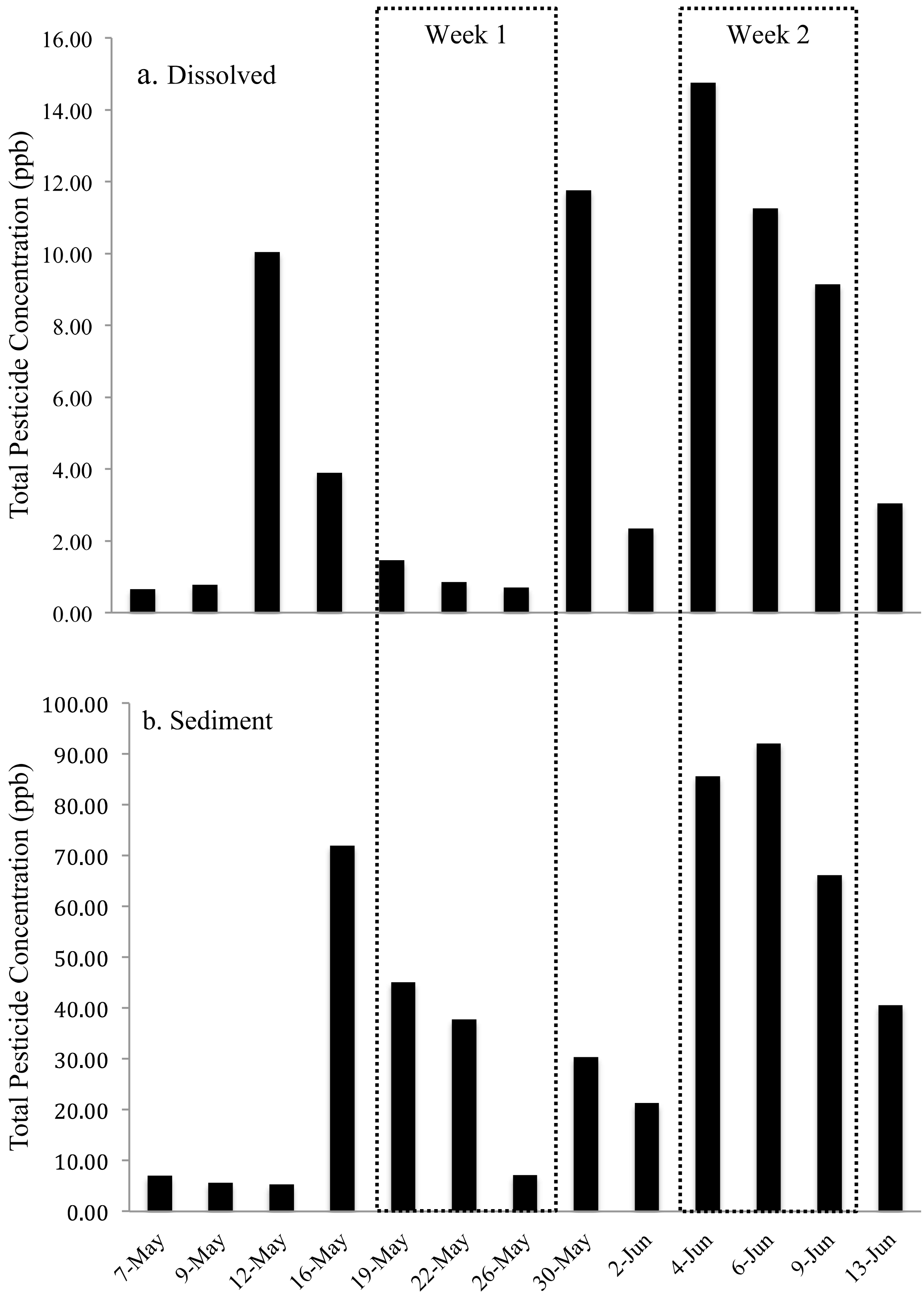

Figure 4. Pesticide concentrations detected in (a) dissolved phase and (b) suspended sediment of Elkhorn River. Boxes indicate the two 7-d fish exposures.

reporting this phenomenon in a mesocosm exposure in environmentally relevant agrichemical profiles.

The demonstrated reduction of estrogen responsive gene expression in the present study is consistent with others performed in the Elkhorn River (Knight et al. 2013; Sellin et al. 2009; Sellin et al. 2010; Ali and Kolok *In press*). As no work has been done on the defeminizing effects of the detected pesticides in our system alone, the down-regulation of *VTG* and *ER*α is most likely be attributed to the effect of a mixture of various endocrine-disrupting agrichemicals. Gordan et al. (2012) supports this hypothesis, noting that no single compound can be held responsible in toxicological studies regarding pulsatile exposures.

With respect to the down-regulation of *AR*, multiple pesticides present in this system have been shown to have anti-androgenic effects in various *in vivo* and *in vitro* models (Cocco, P. 2002; Cooper et al. 200; Sanderson et al. 2000; Thibaut et al. 2004; U.S. Health and Human Services 2003). As with the altered estrogen-responsive gene expression, it is unclear whether one of these chemicals or a chemical mixture was responsible for the observed reduction in expression.

The Impact of Chemical Load and Sediment on Fathead Minnow Gene Expression. During week 2, fish maintained in the whole sediment mesocosm had reduced *VTG* and *ER*α expression with respect to the controls (Figure 2), while those in the fine fraction did not. The lack of defeminization in fish from the fine fraction mesocosm makes it clear that sediment played an important role in the bioavailability of the agrichemicals. It is possible that the sediment in the whole sediment mesocosm served as a sink for contaminants, absorbing them from the water column so that their concentration was too low to elicit an effect in the lower mesocosm. However, previously published work in our lab and that of others confirms that sediment may act as a source of agrichemical contaminants. For example, Sellin et al. (2014) exposed fathead minnows to differing water types with and without sediment taken from the Elkhorn River. Fish exposed to lab water and sediment experienced the same degree of defeminzation as those exposed to river water and sediment. Given these results, we find it unlikely that the whole sediment mesocosm is acting as a sink for water borne chemicals.

Another alternative possibility is that the sediment serves as a slow releasing source of the contaminants. In this scenario, the agrichemicals would desorb into the dissolved phase in the whole sediment mesocosm and be carried down through the water in the cascade. It could then be argued that the contaminant concentrations were not in high enough in the fine fraction mesocosm to cause endocrine disruption. Given the flow rate and volume of each mesocosm, the water from the whole sediment mesocosm would take 40 min to reach the fine fraction mesocosm, which may allow transformation of contaminants and their metabolites into forms that may be less bioavailable. While this is an attractive hypothesis, it may not be applicable to most of the detected agrichemicals in this system, as the aquatic half lives of the top three compounds, atrazine, metalochlor, and acetochlor range from 150-200 days (US HHS 2003; Rivard 2003; Zheng and Yi 2003). An investigation into the impacts of fish in the middle mesocosm would be an appropriate follow-up study for further understanding. Such a study would allow us to see if there is a gradual reduction in agrichemical concentrations, indicated by significant difference in any gene expression among the three mesocosms.

The third scenario is that the sediment is not acting as a source or a sink of agrichemicals, but rather serves as a direct delivery system in which proximity to the sediment is required. It is then possible that the differences between the biological responses of the whole sediment and fine fraction mesocosms are solely due to differences in the total mass of accumulated sediment between the two, as the whole sediment mesocosm contained more than 50 times the amount of sediment as the fine fraction mesocosm (Table 1). Association with a higher mass of sediment-associated contaminants could explain why there was an observed effect in only the whole sediment mesocosm. Fish in this mesocosm had more direct interaction with higher concentrations of sediment via dermal contact, ventilation of turbid water, or ingestion of sediment particles.

Table 1. Total dry mass collected from each mesocosm at the end of each week

MESOCOSM	WEEK 1	WEEK 2
Whole Sediment	60 grams	902 grams
Fine Fraction	1.2 grams	17.8 grams

It is unlikely that ingestion is a significant route of exposure, as determined by previous studies in our laboratory in which fish without direct contact with sediment still experienced changes in gene expression (Jessick et al. 2014). It is also unlikely that the dissolved forms of the contaminants are absorbed through the gills, as this would have caused an effect in both mesocosms. Thus, the results indicate that either direct dermal or gill epithelial contact with the contaminant-laden sediment is most likely responsible for defeminization. Given that the diffusion distances across the skin from water to blood are large due to the fish being covered in scales and mucous, an interaction between the gill epithelium and the sediment seems the most likely route of exposure.

In contrast to the impacts on the estrogen-responsive genes during week 2, the androgen-responsive gene *AR* was significantly down-regulated in both the upper and lower mesocosms, with no significant difference observed between mesocosms (Figure 2). Fish exposed to both the whole sediment and the fine fraction experienced significant reduction in *AR* expression. This suggests that the chemical or chemicals responsible for demasculinization were not associated with the sediment, but rather were in the dissolved phase. The alternative could be that the responsible compound(s) are associated with the sediment, but only low concentrations are necessary to elicit an effect.

Environmental Implications. Taken together, the trends in observed agrichemical concentrations in the dissolved and sediment phases as a function of time show that while the sediment is likely acting as a sink for agrichemicals following precipitation events, and then a source releasing compounds to the dissolved phase, the overall driver for defeminization in this system is direct exposure to the sediment-associated compounds. This study corroborates previous laboratory studies and confirms that sediment plays an important role in the bioavailability of agrichemicals. The results presented in this study are applicable to surface waters located in watersheds where there is significant agricultural production as well as high levels of suspended solids. Risk assessment schemes that focus on the biologic effects of aqueous concentrations of agrichemicals alone are not sufficient, and attention should also be paid to exposure to sediment-associated agrichemicals. These findings may indicate that some agrichemicals may have a greater effect on benthic species compared with pelagic species. However, the present study indicates that fish that are not exclusively benthic, such as fathead minnows, are also affected by direct exposure to contaminated sediments. While the sediment-associated compound was implicated as the driver for defeminization, demasculinization was observed in both the whole sediment and fine fraction mescocosms. This further reinforces that studies evaluating the biological effects due to exposure to complex mixtures of agrichemicals must consider multiple biologic endpoints as well as dissolved and sediment-associated compounds.

CHAPTER 4

THE IMPACT OF EXPOSURE INTERVALS TO AN ENDOCRINE DISRUPTING STEROID AS ITS METABOLITE PROFILE CHANGES

INTRODUCTION

Throughout the United States, bodies of water are contaminated with a variety of endocrine disrupting compounds that enter through surface runoff or groundwater absorption. Manifestations of endocrine disruption include impaired gonadal development, changes in secondary sex characteristics, and increased incidence of intersex fish (Daxenberger 2002; Kloas et al. 2009). Recent studies have also shown significant consequences at the molecular level, specifically in alteration of reproductive gen expression (Sangster et al. 2014; Knight et al. 2013). Endocrine disrupting compounds include pesticides, industrial wastes, and veterinary pharmaceuticals (Kolpin et al. 2002). One such veterinary pharmaceutical is trenbolone acetate, a commonly used growth-promoting hormone in the cattle industry (Ankley et al. 2003). 17-β-trenbolone is one of the metabolites of the biotransformation of this steroid, which is excreted by cattle and enters aquatic systems (Schiffer et al. 2001). This is of particular concern in the Central United States, where approximately half of the land use is dedicated to grazing (Nickerson et al. 2011). Several studies (Soto et al 2004; Orlando et al. 2004; Kolok et al. 2007) have confirmed that sediment from watersheds associated with cattle feedlots contain high levels of this steroid. Although it is not the most prevalent metabolite of trenbolone acetate, 17-β-trenbolone is considered relatively stable in the environment

(Webster et al. 2012) and has the highest binding affinity to the androgen receptor (Schiffer et al. 2001; Ankley et al. 2003). Thus, it is important to understand the fate and transport of this compound in the environment and its biological effect in aquatic systems.

While Ankley et al. (2003) determined that exposure to aqueous 17-β-trenbolone induced defeminization in fathead minnows, little is known about the influence of 17-β-trenbolone and its interaction between sediment and its influence on defeminization. Given the hydrophobic nature of this compound, the dynamic equilibrium between the sediment and water column cannot be ignored. Empirical evidence (Sangter et al. 2014) shows that sediment does indeed influence whether or not a biological effect is observed. Fish exposed to sediment-associated 17-β-trenbolone showed reduced production of the egg-yolk precursor protein vitellogenin (*VTG*), while those exposed to an aqueous solution did not.

In addition to the dynamic sediment-water column equilibrium, the breakdown of 17-β-trenbolone yields a changing composition profile as the compound and its metabolites biotransform and eventually breakdown. Preliminary unpublished data from our lab shows that even low doses of 17-β-trenbolone induce defeminization in just four days. However, fish exposed for a short period of time were able to slightly recover when removed from the contaminated sediment. This implies that the time period and duration to which the fish are exposed affects the degree of defeminization.

The objective of the present study was to determine the impact of exposure intervals to an endocrine disrupting steroid as its metabolite profile in the sediment and water column changes. To achieve this objective, fish were exposed to a controlled in-house sediment and water mixture for various time periods and analyzed for changes in gene expression of two estrogen-responsive genes, vitellogenin (*VTG*) and estrogen-receptor alpha (*ERα*).

MATERIALS AND METHODS:

Test Organism. Sexually mature female fathead minnows (*Pimephales promelas*) from the University of Nebraska at Omaha Aquatic Toxicology Laboratory colony were used for this study. Prior to experimentation, all fish were maintained in 30 L aerated aquaria with dechlorinated lab water, maintained at 25 ± 1°C, and kept under a 16:8 light:dark photoperiod. Fish were fed TetraMin flake food (Aquatic EcoSystems, Apopka, FL, USA) twice daily and received a daily one-third static renewal water change.

Treatment Groups. In order to determine how differences in exposure to a changing metabolite profile would affect the fish, four treatment groups of twelve individuals were developed. The first treatment was controls taken from the general colony. The second was exposed for the first five days after the sediment had been spiked; the third was exposed for ten days after the spiking; and the fourth was added for the last five days of the experiment.

Experimental Design. Sediment for this experiment was collected from the Elkhorn River near Winslow, NE and stored at 4°C until being dried at room temperature for 24 hours before experimental use. A stock of 17-β-trenbolone (98% purity, Sigma, St. Louis, MO, USA) methanol solution was prepared as described in Sangster et al. (2014). One 60 L glass aquarium was divided with a mesh screen and filled with 4 cm of sediment, weighing approximately 13.5 kilograms. One side was for holding fish that would be exposed for 5 days at a time, and the other was to for the 10 day exposed fish. The sediment was spiked with 10 μg of the solution and the solvent allowed to evaporate for

12 hours. Water was then added and stirred for 24 hours prior to adding the fish. Samples of the water were collected every three days and stored at 4°C until analysis. Additionally, daily water temperature, pH (YSI 63, YSI Incorporated, Yellow Springs, OH, USA), dissolved oxygen (DO; YSI 55, YSI Incorporated, Yellow Springs, OH, USA), and turbidity (WQ770-B, Global Water, Gold Water, CA, USA) were measured.

On day one of the experiment, the control treatment fish were lethally anaesthetized with 300 mg/L tricaine methanesulfonate (MS-222; Sigma Aldrich). Body mass was recorded, and liver and gonads were collected, weighed, and flash-frozen in liquid nitrogen. Tissues were stored at -80 ^{o}C until further analysis. Gonadosomatic index (GSI) and hepatosomatic index (HSI) were generated for each individual by dividing the mass of the tissues by the total body mass and multiplying by 100. The same procedure was performed at the end of each treatment's respective exposure period. Once the first 5 day exposed treatment fish were collected, the second 5 day exposed treatment were added to their respective half of the tank.

Real-time PCR and Biological Analysis. Tissues used for quantitative real-time polymerase chain reaction (QPCR) utilized the SV Total RNA Isolation System (Promega, Sunnyvale, CA, USA) and the manufacturer's protocol to extract RNA. The RNA was resuspended and stored in nuclease-free water at -80^{o}C until further analysis. The purity and concentration of the RNA was determined by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). Purity was assessed based on optical densities at 260 nm/230 nm. The samples were diluted to 10 ng/µL in preparation for cDNA synthesis. First-strand cDNA synthesis was performed with 0.75 µg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) in accordance to the manufacturer's recommendations. The QPCRs were performed using the iQ SYBR-Green Supermix (Bio-Rad) in accordance to the manufacturer's protocol. Two µL of diluted cDNA template was added to 300 nM forward and reverse primers in a 13 µL volume containing SYBR-Green Supermix, for a total volume of 15 µL. Two estrogen-responsive genes, *VTG* and *ERα*, were selected for analysis; primer sequences were obtained from Kolok et al., 2007. In addition, the housekeeping gene ribosomal protein L8 was used to normalize gene expression using the $2^{-\Delta C'T}$ method (Livak and Schmittgen 2001).

Data Analysis. Morphometric and relative gene expression data were analyzed by ANOVA followed by Tukey post-hoc comparisons. If assumptions of ANOVA were violated, as was the case with VTG, data were analyzed by Kruskal-Wallis nonparametric comparison followed by a Wilcoxon rank-sum test.

Water Chemical Analysis. Water samples were collected every 3 days and sent to the Water Science Laboratory at the University of Nebraska – Lincoln for analysis. They were screened for the presence of 17-β-trenbolone and its metabolites, 17-α-trenbolone and trendione.

RESULTS

Biological Effects. No changes in body mass, hepatosomatic index, or gonadosomatic index were observed. However, there were notable changes in the hepatic gene expression (Figure 5). Fish exposed during days 0 – 5 had significant reduction in *VTG* compared to controls. The fish exposed for all 10 days and the group exposed from days 5 – 10 did not differ from any of the other treatment groups. Additionally, there was no change in expression of *ERα*.

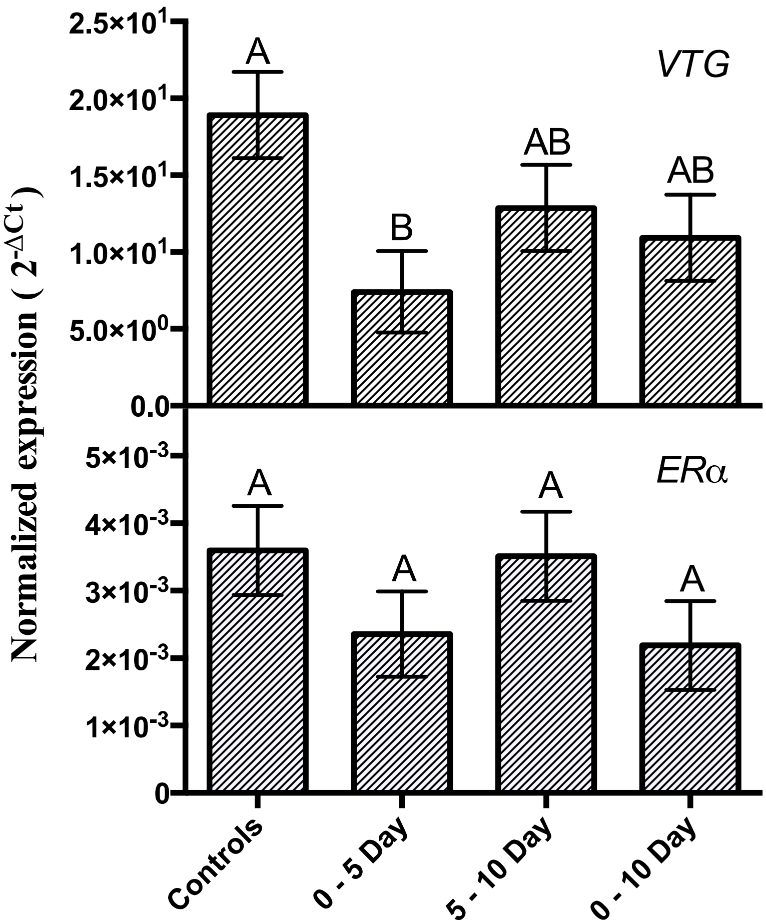

Figure 1. Effects on hepatic mRNA expression of two endocrine responsive genes in female fathead minnows in each treatment group. Significant differences denoted by capital letters as determined by one-way ANOVA or Wilcoxon test. Values represent mean (±SE), sample size of 6-11 fish per treatment group. *VTG* = vitellogenin, *ERα* = estrogen receptor alpha

Water Chemical Analysis. 17-β trenbolone and its metabolites, 17-α-trenbolone and trendione, were below detection limits in all the water samples.

DISCUSSION

The results of this study demonstrate several important things. First, it confirms that exposure to sediment spiked with 17-β-trenbolone causes molecular endocrine disruption. The fish exposed for the first 5 days after sediment spiking were defeminized, showing reductions in *VTG*, while the group that was exposed for 10 days were not. Jessick et al.

(2014) and Sangster et al. (2014) both exposed female fathead minnows to sediment spiked with 17-β-trenbolone, and these animals also experienced reductions in hepatic *VTG* levels, while *ERα* was not affected. Similarly, fish in this study did not show signs of reduced *ERα* expression.

A more important point to consider is the idea that changes due to agrichemical exposure can be activational, having only a temporary effect. Unpublished data in our lab indicates there is a rapid breakdown of 17-β-trenbolone and its metabolites in a laboratory sediment-water setup. After approximately 5 days, these compounds' concentrations in the water are undetectable. Given the observed results of the present study, it is likely that the fish exposed for 10 days did experience defeminization similar to the first treatment of 5-day fish; however, they had additional time to recover in water without contaminants (or at least at very low levels). Thus, it is clear from this study that duration of exposure to an endocrine disruptor is important in the permanency of its effects. This is consistent with unpublished data in our lab (Skolness et al.) in which fish exposed for 4 days recovered after an exposure to sediment spiked with 17-β-trenbolone. An experiment in which 10-day exposed fish were compared to fish taken out of the tank at day 5 and placed in lab water for 5 more days would confirm this hypothesis. Another follow up study could look at the effect of such an exposure on larval fish, as Ali and Farhat (*In review*) suggests that exposure to endocrine disrupting compounds during sexual differentiation may lead to organizational effects, permanently altering the physiology of the fish.

There are environmental implications to consider as well. Previous studies in our lab have detected this compound and its metabolites when spiked at concentrations of 10-900 ng 17-β-trenbolone /g sediment (Jessick et al. 2014; Sangster et al. 2014; Skolness et al. *Unpublished*). The sediment used in this project was spiked at a concentration of 0.75 ng 17-β-trenbolone /g sediment. However, as mentioned previously, the analysis of the water samples did not detect any levels of this steroid or its breakdown products, likely due to such a low dose application. This is important because even at levels below current detection methods, evidence of endocrine disruption is clear. Such a finding suggests that current environmental quality monitoring tools may not detect a problem when there is one. Finally, the impact that cattle feedlots have on the surrounding watershed may be under exaggerated, reaching further downstream than steroid detection alone would predict, and thus should be subject to further studies and scrutiny.

CHAPTER 5

GENERAL CONCLUSIONS, ACKNOWLEDGMENTS, AND REFERENCES

CONCLUSIONS

The studies presented in this thesis confirm that sediment not only has a role in the bioavailability of agrichemicals, but also can be a driving force behind their elicited effects. This point was clearly evident in the first project, in which fish maintained in the whole sediment mesocosm during week 2 had reduced *VTG* and *ER*α expression with respect to the controls, while those in the fine fraction did not (Figure 2). This reveals that the overall driver for defeminization in this system is direct exposure to the sediment-associated compounds. The lack of defeminization in fish from the fine fraction mesocosm makes it clear that sediment played an important role in the bioavailability of the agrichemicals. This idea is also supported when one considers how much lower the sediment levels were in both mesocosms during week 1, where no observable endocrine disruption was seen.

The second study presented here can also illustrate the importance of sediment as a carrier of toxic compounds. Although there was defeminization seen in the fish exposed for the first 5 days (Figure 5), no aqueous 17-β-trenbolone or its metabolites were detected. The hydrophobic nature of 17-β-trenbolone leads to the conclusion that it was present at higher levels in the sediment than the water, where it was still available to fish. The specific uptake mechanism for contaminant-laced sediments is still uncertain, although the results of the first study and others (Jessick et al. 2014) reveal that it is almost certainly dependent on the specific contaminant.

When viewed as a composite, the two studies have considerable environmental implications. First of all these results are applicable to surface waters located in watersheds where there is significant agricultural production as well as high levels of suspended solids. Such systems are prevalent throughout the entire Midwestern region of the United States, where approximately half of land use is developed for agricultural use, and suspended sediment levels are much higher than other regions of the country (Lee and Glyson 2013; Pryor et al. 2013). Risk assessment focusing on the biological effects of aqueous concentrations of agrichemicals alone may not be sufficient, and attention should also be paid to exposure to sediment-associated agrichemicals. These findings may indicate that some agrichemicals may have a greater effect on benthic species compared with pelagic species. However, the present study indicates that fish that are not exclusively benthic, such as fathead minnows, are also affected by direct exposure to contaminated sediments. While the sediment-associated compound was implicated as the driver for defeminization, demasculinization was observed in both the whole sediment and fine fraction mesocosms. This further reinforces that studies evaluating the biological effects due to exposure to complex mixtures of agrichemicals must consider multiple biological endpoints as well as dissolved and sediment-associated compounds.

Another point for consideration is that the stage to which animals are exposed may have an impact on the severity of endocrine disruption they experience. The 10-d adults in the second study were able to rapidly recover after a brief exposure to a relatively low dose of contaminant. It is clear that for this particular compound, activational effects, such as reversible changes in gene expression, can be seen even when the contaminant level is undetectable, which is in and of itself a concern. However, further studies are necessary to determine the applicability of this study, as the effect may be even greater in larval stages. Preliminary evidence (Ali and Farhat *In review*) shows that a 7-d exposure to an agrichemical pulse during sexual differentiation (5 days post hatch) causes observable endocrine disruption, which lasts for at least several months. Thus, the recovery seen in the present study may not necessarily be consistent to all life stages of aquatic organisms. Such a hanging question opens to door to extensive follow-up studies on the long-term effects of contaminant exposures.

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REFERENCES

Ali, J. M.; Kolok, A.S. On-site serial exposure of female fathead minnows to the Elkhorn River, NE, USA spring agrichemical pulse. *Environ Tocicol Chem*, *In press*.

Ali, J; Farhat, Y. Altered aromatase expression in larval fathead minnows following exposure to an agrichemical pulse. *Environ Toxicol Chem*, *In review*.

Ankley, G.T.; Kahl, M.D.; Jensen, K.M.; Hornung, M.W.; Korte, J.J.; Makynen, E.A.; Leino, R.L. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). *Toxicol Sci*, **2002**, *67*, 121-130.

Ankley, G.T.; Jensen, K.M.; Makynen, E.A.; Kahl, M.D.; Korte, J.J.; Hornung, M.W.; Henry, T.R.; Denny, J.S.; Leino, R.L.; Wilson, V.S.; Cardon, M.C.; Hartih, P.C.; Gray, L.E. Effects of the androgenic growth promoter 17-β-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. *Environ Toxicol Chem*, **2002**, *22*(6), 1350-1360.

Arcand-Hoy, L.D.; Benson, W.H. Fish reproduction: an ecologically relevant indicator of endocrine disruption. *Environ Toxicol Chem*, **1998**, *17*(1), 49-57.

Barnes, K.K.; Christenson, S.C.; Kolpin, D.W.; Focazio, M.; Furlong, E.T.; Zaugg, S.D.; Meyer, M.T.; Barber, L.B. Pharmaceuticals and other organic waste water contaminants within a leachate plume downgradient of a municipal landfill. *Ground Water Monit Rem*, **2004**, *24*(2), 119-126.

Barber, L.B. Vajda, A.M., Douville, C., Norris, D.O. and Writer, J.H. Fish endocrine disruption responses to a major wastewater treatment facility upgrade. *Environ Sci Technol*, **2012**, *46,* 2121-2131.

Benotti, M.J.; Trenholm, R.A.; Vanderford, B.J.; Holady, J.C.; Stanford, B.D.; Snyder, S.A. Pharmaceuticals and endocrine disrupting compounds in U.S. drinking water. *Environ Sci Technol*, **2009**, *43*, 597-603.

Borton, D.L.; Cook, D.L; Bradley, W.K.; Philbeck, P.E.; Dubé, M.G.; Brown-Peterson, N.J.; Streblow, W.R. Responses of fathead minnows (*Pimephales promelas*) during life-cycle exposures to pulp mill effluents at four long-term receiving water study sites. *Integr Environ Assess Manag*, **2009**, *5*(2), 270-282.

Carter, J.; Walling, D.E.; Owens, P.N.; Leeks, G.J.L. Spatial and temporal variability in the concentration and speciation of metals in suspended sediment transported by the River Aire, Yorkshire, UK. *Hydrol. Process.* **2006,** *20* (14), 3007-3027

Chapman, A.S.; Foster, I.D.L.; Lees, J.A.; Hodgkinson, R.A. Sediment delivery from agricultural land to rivers via subsurface drainage. *Hydrol Process*, **2005**, *19*, 2875-2897.

Cocco, P. On the rumors about the silent spring: review of the scientific evidence linking occupational and environmental pesticide exposure to endocrine disruption health effects. *Cadernos de Saúde pública*, **2002**, *18*(2), 379-402.

Colborn, T.; von Saal,, F.S.; Soto, A.M.. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect*, **1993**, *101*(5), 378-384.

Cooper, R.; Stoker, T.; Tyrey, L.; Goldman, J.; McElroy, W. Atrazine disrupts the hypothalamic control of pituitary-ovarian function. *Toxicol. Sci.* **2000,** *53* (2), 297-307.

Coupe, R.; Henebry, M.; Branham, M. Assessing the Effect of Pesticides in Agricultural Runoff on Aquatic Life in the Sangamon River Near Monticello, Illinois. *Water Sci. Technol.* **1993,** *28* (3-5), 569-573

Daughton, C.G.; Ternes, T.A. Pharmacueticals and personal care products in the environment: agents of subtle change? *Environ Health Perspect*, **1999**, *107*(S6), 907-938.

Decision documents for atrazine. U.S. Environmental Protection Agency: Washington, D.C. **2006**.

http://www.epa.gov/pesticides/reregistration/REDs/atrazine_combined_docs.pdf.

Daxenberger, A. Pollutants with androgen-disrupting potency. *Eur J Lipid Sci Technolci,* **2002**, *104*, 124-130.

Duong, C.N.; Schlenk, D.; Chang, N.I.; Kim, S.D. The effect of particle size on the bioavailability of estrogenic chemicals from sediments. *Chemosphere*, **2009**, *76*(3), 395-401.

Falconer, I.R.; Chapman, H.F.; Moore, M.R.; Ranmuthugala, G. Endocrine-disrupting compounds: a review of their challenge to sustainable and safe water supply and water reuse. *Environ Toxicol*, **2006**, *21*, 181-191.

Filby, A.L.; Thorpe, K.L.; Maack, G.; Tyler, C.R. Gene expression profiles revealing the mechanisms of anti-androgen-and estrogen-induced feminization in fish. *Aquat. Toxicol.* **2007,** *81* (2), 219-231

Glassmeyer, S.T.; Furlong, E.T.; Kolpin, D.W.; Chaill, J.D.; Zaugg, S.D.; Werner, S.L.; Meyer, M.T.; Kryak, D.D. Transport of chemical and microbial compounds from known wastewater discharges: potential for use as indicators of human fecal contamination. *Environ Sci Technol*, **2005**, *39*, 5157-5169.

Gordan, A.K.; Mantel, S.K.; Muller, M.W.J. Review of toxicological effects caused by episodic stressor exposure. *Environ. Toxicol. Chem*. **2012,** *31* (5), 1169-1174

Hutchinson, T.H.; Ankley, G.T.; Segner, H.' Tyler, C.R. Screening and testing for endocrine disruption in fish – biomarkers as "signposts," not "traffic lights," in risk assessment. *Environ Heal Perspect*, **2006**, *114*(S1), 106-114.

Hyer, K.; Hornberger, G.; Herman, J. Processes controlling the episodic streamwater transport of atrazine and other agrichemicals in an agricultural watershed. *J. Hydrol.* **2001,** *254* (1-4), 47-66.

Iguchi, T.; Katsu, Y. Commonality in signaling of endocrine disruption from snail to human. *Bioscience*, **2008**, *58*(11), 1061-1067.

Izquierdo, M.; Tye, A.M.; Chenery, S.R. Sources, lability and solubility of Pb in alluvial soils of the River Trent catchment, UK. *Sci. Total Environ.* **2012,** *433*, 110-122.

Jenkins, R.; Wilson, E.; Angus, R.; Howell, W.; Kirk, M. Androstenedione and progesterone in the sediment of a river receiving paper mill effluent. *Toxicol. Sci.* **2003,** *73* (1), 53-59.

Jansen, M.S.; Nagel, S.C.; Miranda, P.J.; Lobenhofer, E.K.; Afshari, C.A.; McDonnell, D.P. Short-chain fatty acids enhance nuclear receptor activity through mitogen-activated protein kinase activation and histone deacetylase inhibition. *Proc Natl Acad Sci*, **2004**, *101*(18), 7199-7204.

Jessick, A.M.; Skolness, S.; Kolok, A.S. Sandy sediment and the bioavailability of 17 beta-trenbolone to adult female fathead minnows. *Aquat. Toxicol.* **2014,** *148*, 48-54.

Kloas, W.; Urbatzka, R.; Opitz, R.; Würtz, S., Behrends, T.; Hermelink, B.; Hofmann, F.; Jagnytsch, O.; Kroupova, H.; Lorenz, C.; Neumann, N.; Pietsch, C.; Trubiroha, A.; van Ballegooy, C.; Wiedemann, C.; Lutz, I. Endocrine disruption in aquatic vertebrates. *Ann NY Acad Sci*, **2009**, *1163*, 187-200.

Knight, L.A.; Christensen, M.K.; Trease, A.J.; Davis, P.H.; Kolok, A.S. The spring runoff in Nebraska's (USA) Elkhorn River watershed and its impact on two sentinel organisms. *Environ Toxicol Chem, **2013**, 32*(7), 1544-1551.

Kolok, A.S.; Snow, D.D.; Kohno, S.; Sellin, M.K.; Guillette Jr., L.J. Occurrence and biological effect of exogenous steroids in the Elkhorn River, Nebraska, USA. *Sci Total Environ*, **2007**, *388*, 104-115.

Kolpin, D.W.; Furlong, E.T.; Meyer, M.T.; Thurman, E.M.; Zaugg, S.D.; Barber, L.B.; Buxton, H.T. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. Streams, 1999-2000: a national reconnaissance. *Environ Sci Technol*, **2002**, *36*, 1202-1211.

Kronvang, B.; Laubel, A.; Grant, R. Suspended sediment and particulate phosphorus transport and delivery pathways in an arable catchment, Gelbaek Stream, Denmark. *Hydrol. Process.* **1997,** *11* (6), 627-642.

Krutz, L.J.; Senseman, S.A.; Zablotowicz; Matocha, M.A. Reducing herbicide runoff from agricultural fields with vegetable filter strips: a review. *Weed Sci*, **2005**, *53*, 353-367.

Lee, C.J.; Glysson, G.D. Compilation, quality control, analysis, and summary of discrete suspended-sediment and ancillary data in the United States, 1901-2010; *U. S. Geological Survey Data Series 776*; U.S. Geological Survey: Reston, VA, **2013**; http://pubs.usgs.gov/ds/776/pdf/ds776.pdf

Lee, K.E., Barber, L.B., and Schoenfuss, H.L. Spatial and temporal patterns of endocrine active chemicals in small streams indicate differential exposure to aquatic organisms. *J Am Water Resour As*, **2014**, *50*(2), 401-419.

Li, Y; Luh, C.J.; Burns, K.A.; Arao, Y; Jiang, Z.; Teng, C.t.; Tice, R.R.; Korach, K.S. Endocrine-disrupting chemicals (EDCs): *in vitro* mechanisms of estrogenic activation and differential effects on ER target genes. *Environ Heal Perspect*, **2013**, *121*(4), 459-466.

Liu, Z.; Kanjo, Y.; Mizutani, S. Removal mechanisms for endocrine disrupting compounds (EDCs) in wastewater treatment – physical means, biodegradation, and chemical advanced oxidation: a review. *Sci Total Environ*, **2009**, *407*, 731-748.

Livak, K. and Schmittgen, T. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* **2001,** *25* (4), 402-408.

Lye, C.M.; Frid, C.L.J.; Gill, M.E.; McCormick, D. Abnormalities in the reproductive health of flounder *Platichthys flesus* exposed to effluent from a sewage treatment work. *Marine Pollut Bull*, **1997**, *34*(1), 34-41.

Martinović, D.; Blake, L.B.; Durhan, E.J.; Greene, K.J.; Kahl, M.D.; Jensen, K.M.; Makynen, E.A.; Villeneuve, D.L.; Ankley, G.T. Reproductive toxicity of vinclozolin in the fathead minnow: confirming an anti-androgenic mode of action. *Environ Toxicol Chem*, **2007**, *27*(2), 478-488.

McKinlay, R.; Plant, J.A.; Bell, J.N.B.; Voulvoulis, N. Endocrine disrupting pesticides: implications for risk assessment. *Environ. Int.* **2008,** *34* (2), 168-183.

Munger, R; Isacson, P.; Hu, S.; Burns, T.; Hanson, J.; Lynch, C.F. Intrauterine growth retardation in Iowa communities with herbicide-contaminated drinking water supplies. *Environ Heal Perspect*, **1997**, *105*(3), 308-314.

Norris, D.O.; Carr, J.A. *Biological Bases for Health Effects in Wildlife and Humans*. **2006**, Oxford University Press, New York, New York.

Orlando, E.F.; Kolok A.S.; Binzcik, G.A.; Gates, J.L.; Horton, M.K.; Lambright, C.S.; Gray, Jr., L.E.; Soto, A.M.; Guillette, Jr., L.J. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. *Environ Health*

Perspect, **2004,** Mar;*112*(3), 353–358.

Orrego, R., Guchardi, J., Hernandez, V., Krause, R., and Roti, L. Pulp and paper mill effluent treatments have differential endocrine-disrupting effects on rainbow trout. *Environ Toxicology Chem*, **2009**, *28*(1), 181-188.

Ouyang, W.; Huang, H.; Hao, F.; Shan, Y.; Guo, B. Evaluating spatial interaction of soil property with non-point source pollution at watershed scale: The phosphorus indicator in Northeast China. *Sci Total Environ.* **2012,** *432*, 412-421.

Parrott, J.L.; Blunt, B.R. Life-cycle exposure of fathead minnow (*Pimephalus promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. *Environ Toxicol*, **2005**, *20*(2), 131-141.

Pereira, W.E.; Rostad, C.E. Occurrence, distributions, and transport of herbicides and their degradation products in the lower Mississippi River and its tributaries. *Environ Sci Technol*, **1990**, *24*(9), 1400-1408.

Petrović, M.; Gonzalez, S.; Barceló, D. Analysis and removal of emerging contaminants in wastewater and drinking water. *Trends Anal Chem,* **2003**, *22*(10), 685-696.

Pickering, A.D.; Sumpter, J.P. Comprehending endocrine disruptors in aquatic environments. *Environ Sci Technol*, **2003**, *37*(17), 331A-336A.

Pryor, S.C.; Barthelmie, R.J.; Schoof, J.T. High-resoltion projections of climate impacts for the Midwestern USA. *Climate Res*, **2013**, *56*, 61-79.

Razin A.; Kantor, B. DNA methylation in epigenetic control of gene expression. *Prog Mol Subcell Biol*, **2005**, *38*, 151-167.

Sanderson, J.; Seinen, W.; Giesy, J.; van den Berg, M. 2-chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: A novel mechanism for estrogenicity? *Toxicol. Sci.* **2000,** *54* (1), 121-127.

Sangster, J.L.; Zhang, Y.; Hernandez, R.; Garcia, Y.A.; Sivils, J.C.; Cox, M.B.; Snow, D.D.; Kolok, A.S.; Bartelt-Hunt, S.L. Bioavailability and fate of sediment-associated trenbolone and estradiol in aquatic systems. *Sci. Total Environ.* **2014,** *496*, 576-584.

Schiffer, B.; Daxenberger, A.; Meyer, K.;Meyer, H.H.D. The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: environmental studies. *Environ Heal Perspect*, **2001**, *109*911), 1145-1151.

Schlenk, D.; Sapozhnikova, Y.; Irwin, M.A.; Xie, L.; Hwang, W.; Reddy, S.; Brownawell, B.J.; Armstrong, J.; Kelly, M.; Montagne, D.E.; Kolodziej, E.P.; Sedlak, D.; Snyder, S. In vivo bioassay-guided fractionation of marine sediment extracts from the southern California Bight, USA, for estrogenic activity. *Environ Toxicol Chem*, **2005**, *24*(11), 2820-2826.

Sellin, M.K.; Snow, D.D.; Gustafson, S.T.; Erickson, G.E.; Kolok, A.S. The endocrine activity of beef cattle wastes: Do growth-promoting steroids make a difference? *Aquat. Toxicol.* **2009**, *92* (4), 221-227.

Sellin, M.K.; Snow, D.D.; Kolok, A.S. Reductions in hepatic vitellogenin and estrogen receptor alpha expression by sediments from an agriculturally impacted waterway. *Aquat Toxicol*, **2010**, *96*, 103-108.

Soares, A.; Guieysse, B.; Jefferson, B.; Cartmell, E.; Lester, J.N. Nonylphenol in the environment: A critical review on occurrence, fate, toxicity and treatment in wastewaters. *Environ. Int.* **2008,** *34* (7), 1033-1049.

Soto, A.M.; Prechti, N.V.; Yau, A.Y.; Orlanda, E.F.; Daxenberger, A.; Kolok, A.S.; Gullette, Jr., L.J.; le Bizec, B.; Lange, I.G.; Sonnenschein, C. Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in Eastern Nebraska, USA. *Environ Heal Perspect*, **2004**, *122*(3), 346-352.

Sumpter, J.P. Xenoendocrine disrupter – environmental impact. *Toxicol Lett*, **1998**, *102-103*(28), 337-342.

Tabb, M.M.; Blumber, B. New modes of action for endocrine-disrupting chemicals. *Mol Endocrinol*, **2006**, *20*(3), 475-482.

Thibaut, R. and Porte, C. Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. *J. Steroid Biochem. Mol. Biol.* **2004,** *92* (5), 485-494.

Thomann, R.V.; Connolly, J.P.; Parkerton, T.F., 1992. An equilibrium model of organic chemical accumulation in aquatic food webs with sediment interaction. *Environ Toxicol.* Chem,**1992**,*11*, 615-629.

Thorpe, K.L., Maack, G., Benstead, R. and Tyler, C.R. Estrogenic wastewater treatment works effluents reduce egg production in fish. *Environ Sci Technol*, **2009**, *43*, 2976-2982.

Thurman, E.; Goolsby, D.; Meyer, M.; Kolpin, D. Herbicides in Surface Waters of the Midwestern United States - the Effect of Spring Flush. *Environ Sci Technol* **1991,** *25* (10), 1794-1796.

Total maximum daily loads for the Elkhorn River Basin. United States Nebraska Department of Environmental Quality Planning Unit, Water Quality Division: Lincoln, NE.,**2009**; http://deq.ne.gov/NDEQProg.nsf/xsp/.ibmmodres/domino/OpenAttachment/NDEQProg.nsf/D39AC9D9885669C286257BF80050B95F/Body/elkhorn%20stream%20tmdls.pdf

Toxicological profile for atrazine; CAS# 1912-24-9; U.S. Department of Health and Human Services, Public Health Services: Atlanta, GA. **2003**; http://www.atsdr.cdc.gov/toxguides/toxguide-153.pdf.

Tyler, C.R.; Jobling, S.; Sumpter, J.P. Endocrine disruption in wildlife: a critical review of the evidence. *Crit Rev Toxicol*, **1998**, *28*(4), 319-361.

U. S. Geological Survey 06800500 Elkhorn River at Waterloo, NE; http://waterdata.usgs.gov/usa/nwis/uv?site_no=06800500.

Vajda, A.M.; Barber, L.B.; Gray, J.L.; Lopez, E.M.; Woodling, J.D.; Norris, D.O. Reproductive disruption in fish downstream from an Estrogenic wastewater effluent. *Environ. Sci. Technol.* **2008,** *42* (9), 3407-3414.

Van den Heuvel, M.R. Recent progress in understanding the causes of endocrine disruption related to pulp and paper mill effluents. *Water Qual Res J Can*, **2010**, *45*(2), 137-144.

Wauchope, R.D. The pesticide content of surface water draining from agricultural fields: a review. *J Environ Qual*, **1978**, *7*(4), 459-472.

Webster, J.P., Kover, S.C., Bryson, R.J., Harter, T., Mansell, D.S., Sedlak, D.L., and Kolodziej. Occurrence of trenbolone acetate metabolites in simulated confined animal feeding operation (CAFO) runoff. *Environ Sci Tecnol*, **2012**, *46*(7), 3803-3810.

Writer, J.H.; Barber, L.B.; Ryan, J.N.; Bradley, P.M. Biodegradation and Attenuation of Steroidal Hormones and Alkylphenols by Stream Biofilms and Sediments. *Environ. Sci. Technol.* **2011,** *45* (10), 4370-4376.

Yang, S.; Carlson, K. Evolution of antibiotic occurrence in a river through pristine, urban, and agricultural landscapes. *Water Res*, **2003**, *37*(19), 4645-4656.

Zhang, Y.; Krysl, R.G.; Ali, J.M.; Snow, D.D.; Bartelt-Hunt, S. L.; Kolok, A.S. Impact of sediment on agrichemical fate and bioavailability to adult fathead minnows: a field study. *Environ Sci Tech*, *In review*.

Zhang, Y.; Zhou, J.L. Occurrence and removal of endocrine disrupting chemicals in wastewater. *Chemosphere*, **2008**, *73*(5), 848-853.

Zou, E. Impacts of xenobiotics on crustacean molting: the invisible endocrine disruption. *Integr Comp Biol*, **2005**, *45*, 33-38.