

ABSTRACT

Title of Thesis: APPROPRIATENESS OF LARGEMOUTH BASS AS A MODEL SPECIES FOR DETECTION OF ENDOCRINE DISRUPTION

Christine Kim, Angela Leasca, Winston Liu, Shivani Patel, Laura Poulsen, Shefali Shah, Taylor Throwe, Renuka Tripu

Thesis directed by: Dr. Lance Yonkos
Department of Environmental Science & Technology

Intersex in largemouth bass (*Micropterus salmoides*) has been correlated with regional anthropogenic activity, but has not been causally linked to environmental factors. Four groups of hatchery-reared largemouth bass (LMB) and fathead minnows (FHM) of varying ages and sex were exposed to aqueous poultry litter mixtures, 17 β -estradiol (E2), and controls. Water samples were analyzed for estrogens through liquid chromatography tandem mass spectrometry and estrogenicity through the bioluminescent yeast estrogen screen assay. Fish plasma was analyzed for the egg yolk protein vitellogenin (Vtg) using enzyme-linked immunosorbent assay and gonad tissue was examined histologically for enumeration of testicular oocytes (TO). Water chemistry revealed typical E2 conversion to Estrone with subsequent decay over the exposure periods. A modest prevalence of TO (9.4%) was detected with no apparent treatment effect. While significant Vtg induction was found in E2 exposed FHM, minimal Vtg induction was found in male LMB. Despite field findings of intersex in male LMB, this species may be poorly suited for laboratory investigations into endocrine disruption.

Key Words: Endocrine disruption; Intersex; Testicular oocytes; Vitellogenin;

Largemouth bass; Poultry litter; Fathead minnow; Estradiol

APPROPRIATENESS OF LARGEMOUTH BASS AS A MODEL SPECIES FOR
DETECTION OF ENDOCRINE DISRUPTION

By Team Bass

Christine Kim, Angela Leasca, Winston Liu, Shivani Patel, Laura Poulsen, Shefali Shah,
Taylor Throwe, Renuka Tripu

Dr. Lance Yonkos

Thesis submitted in partial fulfillment of the Gemstone Program
University of Maryland
2015

Advisory Committee:

Professor Lance Yonkos, Mentor
Mr. Steven Turley
Dr. Ed Orlando
Dr. Fred Pinkney
Dr. Beth McGee
Dr. Scott Lynn
Dr. Vicki Blazer
Dr. Luke Iwanowicz

Acknowledgements

First and foremost, we would like to thank our mentor, Dr. Lance Yonkos for his guidance, knowledge, patience, and time. We would like to thank our discussants: Mr. Steven Turley, Dr. Ed Orlando, Dr. Fred Pinkney, Dr. Beth McGee, Dr. Scott Lynn, Dr. Vicki Blazer, and Dr. Luke Iwanowicz. We would also like to thank Dr. Diana Aga and Elizabeth Mullen for estrogen quantification; Jud Blazek and Stuart Lehmann from the DNR Cooperative Oxford Lab for histology preparation; Mark Matsche, Dr. Luke Iwanowicz, and Heather Walsh for teaching us new methods; Dr. L. Curry Woods and Dan Theisen for providing laboratory space; Dr. Joseph Love for providing fish; Elizabeth Friedel, Alex MacLeod, Sharon Hartzell, and Kirsten Hammett for laboratory assistance; and Dr. Douglass Powell from IACUC. We are grateful for the assistance from our librarian Ms. Nevenka Zdravkovska. Lastly, we would like to thank the Gemstone Program, Dr. Frank Coale, Dr. Kristan Skendall, Dr. James Wallace, Dr. Rebecca Thomas, and Vickie Hill for their support and guidance over the past four years.

Table of Contents

Acknowledgements	ii
Table of Contents	iii
1. INTRODUCTION	1
2. LITERATURE REVIEW	5
2.1. Largemouth Bass (<i>Micropterus salmoides</i>)	5
2.2. Sex Determination in Largemouth Bass	8
2.3. The Effects of EDCs	11
2.3.1. Windows of Sensitivity	12
2.4. Poultry Litter as a Source of EDCs	15
2.5. Fathead Minnow (<i>Pimephales promelas</i>)	18
3. MATERIALS AND METHODS	20
3.1. Experimental Overview	20
3.1.1. Exposure I: 12 mph LMB	21
3.1.2. Exposure II: 3 mph LMB	21
3.1.3. Exposure III: Multi-species Comparison of Sensitivity to Endocrine Disruption	21
3.1.4. Exposure IV: Effect of Estradiol Concentration on Sensitivity to Endocrine Disruption	22
3.2. Fish Cohort Maintenance	23
3.2.1. Acquisition of fish	23
3.2.2. Largemouth Bass Housing	24
3.3. Exposure Set-up	25
3.3.1. Poultry Litter and Estradiol Exposure Media Preparation	25
3.3.2. Laboratory Set-up	26
3.4. Water Chemistry	28
3.4.1. Water Sample Collection & Preparation	28
3.4.1.1. Extraction Process for BLYES Estrogenicity Assessment	30
3.4.1.2. Extraction Process for Estrogen Quantification by LC MS/MS	30
3.4.2. Estrogen Measurement using LC MS/MS	31
3.4.3. Estrogenicity Measurement using BLYES	32
3.5. Biological Sample Collection and Analysis	34
3.5.1. Fish Sacrifice and Plasma Collection	34
3.5.2. ELISA Vitellogenin Measurement	35
3.5.3. Fish Gonad and Tissue Collection	36
3.5.4. Testicular Oocyte Detection through Histological Examination	38
3.6. Statistical Analysis	39
4. RESULTS	39
4.1. Survival	39
4.2. Estrogenicity by BLYES	40
4.2.1. Exposure I BLYES Results	41
4.2.2. Exposure II BLYES Results	41
4.2.3. Exposure III BLYES Results	43

4.2.4. Exposure IV BLYES Results	43
4.3. Estrogen Quantification by LC MS/MS	44
4.3.1. Exposure III Estrogen Quantification by LC MS/MS	45
4.3.2. Exposure IV Estrogen Quantification by LC MS/MS	47
4.4. Plasma Vitellogenin Measured Using ELISA	48
4.4.1. Exposure I Vtg Results	49
4.4.2. Exposure II Vtg Results	50
4.4.3. Exposure III Vtg Results	50
4.4.4. Exposure IV Vtg Results	51
4.5. Histology	53
5. DISCUSSION	55
BIBLIOGRAPHY	62
Appendices	69
Appendix A: Bioluminescence Yeast Estrogen Assay	69
Appendix B: Enzyme-Linked Immunosorbant Plasma Vitellogenin Assay (ELISA) ..	73
Appendix C: Incidence of TO in Grow-Out LMB	77
Appendix D: University of Maryland Crane Aquaculture Facility Layout	78
Appendix E: Overview of Exposures I-IV	79
Appendix F: Histological Documentation of TO in LMB	81
Appendix G: University of Maryland Animal Sciences Aquaculture Facility	84
Appendix H: Fish Plasma and Gonad Collection	85

1. INTRODUCTION

The Chesapeake Bay Watershed is a complex ecological system that provides habitat for a variety of organisms, including *Micropterus dolomieu* (smallmouth bass) and *Micropterus salmoides* (largemouth bass), collectively known as black bass. In 2002, fish kills were observed among smallmouth bass throughout the south branch of the Potomac River, a tributary of the Chesapeake Bay (Blazer et al., 2007) (Figure 1). Histological investigation of black bass collected within affected regions revealed oocytes, or female germ cells, in nearly all of the male testes. The presence of testicular oocytes (TO), one of several conditions referred to as intersex, has been documented as a national problem, especially in black bass (Hinck et. al, 2009). Aqueous exposure to endocrine disrupting compounds (EDCs) is associated with TO development (Blazer et al., 2007). Studies of urban and industrial EDCs such as sewage effluent and paper mill waste have shown significant correlations with TO development (Pait & Nelson, 2003). In addition, discharge from wastewater treatment plants has been shown to induce vitellogenesis in male bass (Jobling et al., 1998). Vitellogenin (Vtg) is a female egg precursor protein not normally expressed in males and can therefore be used as an indicator of endocrine disruption (Sumpter & Jobling, 1995). However, in agricultural areas, with little to no industrial EDC sources, male black bass still exhibit high prevalence of TO development and vitellogenesis, suggesting that agricultural runoff may also cause endocrine disruption.

Poultry litter is used extensively as a fertilizer because it is nutrient rich, easily accessible, and inexpensive. The lower Eastern Shore of Maryland alone produces 1.6 billion pounds of poultry litter annually (Agriculture, 2002). Runoff from agricultural

poultry litter application has been shown to be rich in a variety of EDCs, most notably the natural estrogens 17 β -estradiol (E2) and estrone (E1) (Nichols et al., 1997). These and other *poultry litter-associated compounds* (PLACs) have the potential to cause intersex in fish populations (Yonkos et al., 2010), including the largemouth bass.

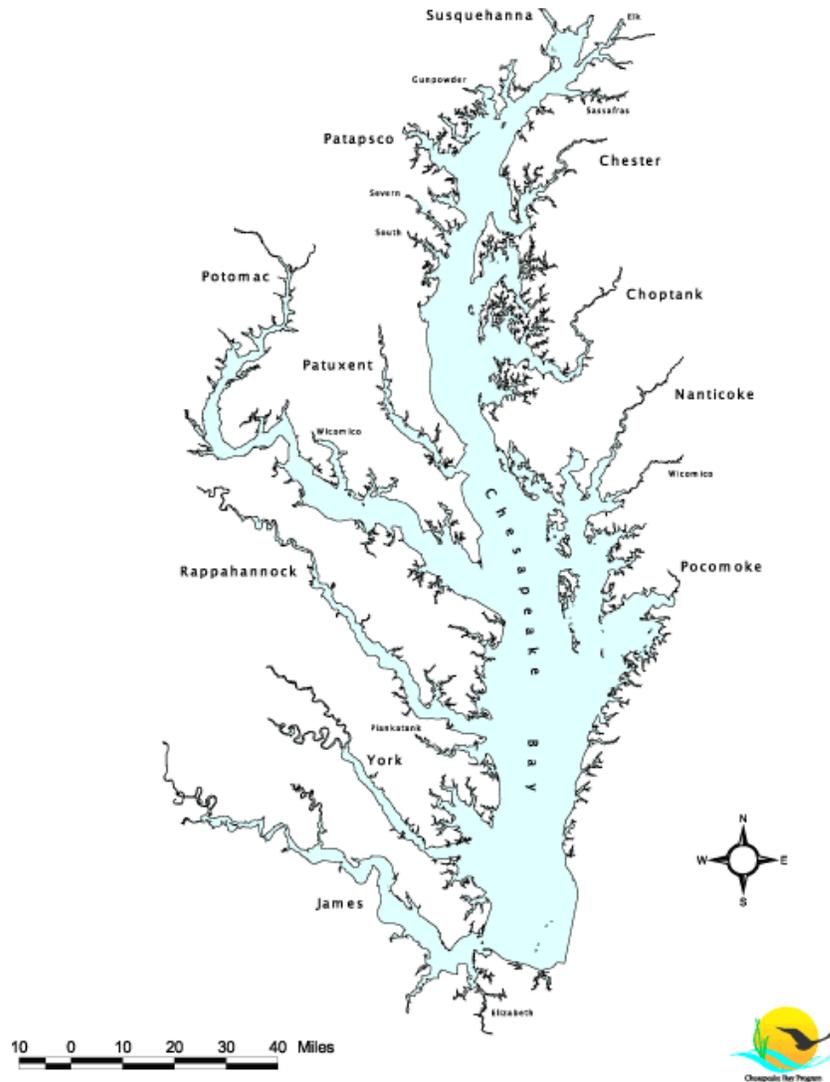


Figure 1. Chesapeake Bay tributaries.

Instances of TO in male largemouth bass within the Chesapeake watershed have been observed near Potomac River wastewater treatment plants (23%; Iwanowicz et al., 2009) and within agriculturally intensive regions of the Eastern Shore (33% to 88%;

Yonkos et al., 2010). However, studies of largemouth bass exposed to poultry litter have not been reported in the scientific literature. Because the Potomac River serves as source water for municipal drinking water, observed intersex in regional fish raises the possibility that EDCs within the system could also have human health implications. As the Chesapeake Bay watershed spans 64,000 square miles and provides drinking water to seventeen million people, further study of the effects of EDCs is imperative.

The goal of our research was directed toward understanding the relationship between EDCs originating in poultry litter and the occurrence of intersex in largemouth bass within Chesapeake Bay tributaries. We chose to study largemouth bass because they are economically and ecologically important within many freshwater portions of the Chesapeake watershed and have documented regional occurrences of intersex (MD DNR, 2007). However, while the species is amenable to handling and confinement, the species has the disadvantage of slow maturation and substantial space requirements (MD DNR, 2007). Our specific research aims were to answer the following questions:

- 1) Can exposure to aqueous poultry litter extracts induce testicular oocytes (TO) and/or vitellogenin (Vtg) in male largemouth bass?
- 2) Is there an age, window of sensitivity, during which largemouth bass are most sensitive to endocrine disruption?
- 3) How does the endocrine sensitivity of the largemouth bass compare to that of the fathead minnow (FHM) (*Pimephales promelas*)?

We hypothesized that poultry litter effluent is responsible for intersex in largemouth bass in Chesapeake Bay estuaries, and therefore, that aqueous exposure of largemouth bass to estrogen-containing media would induce biomarkers indicative of

feminization. Specifically, TO would be induced to greater prevalence in male fish and the egg yolk protein vitellogenin (Vtg) would be induced in male fish. We predict that these responses would occur in largemouth bass in a developmental age-dependent manner and that largemouth bass would have a similar sensitivity to aqueous estrogenic exposures as that reported for the common laboratory fish, the fathead minnow. Our null hypotheses are 1) Poultry litter will not significantly increase the severity or prevalence of endocrine disruption 2) There will no difference in sensitivity to EDCs across the different age groups we chose to study and 3) There will be no difference in endocrine sensitivity between FHM and LMB.

To test the first two hypotheses, we performed a series of laboratory experiments exposing largemouth bass to aqueous poultry litter extracts and to potentially-estrogenic E2 positive control treatments at four different ages (approximately 3 months post hatch (mph), 6 mph, 12 mph, and 18 mph). Subsamples of the exposed fish were examined immediately after the 14-d exposure intervals to investigate Vtg induction and after prescribed grow-out intervals of 3 to 9 months to examine prevalence and severity of TO. We predicted that the percent of male bass with TO would be higher in groups exposed either to poultry litter or the estradiol positive control than in the unexposed group (negative control). Likewise, we predicted that plasma Vtg levels would be higher in male bass exposed either to poultry litter or the positive control than in unexposed bass. In addition, we predicted that for a given dose of poultry litter, the percentage of bass with TO would be higher for fish exposed to poultry litter at a younger age (e.g., during gonadal differentiation) than for older, more mature fish. However, limited

availability of fish from the state run hatchery and limitations in laboratory facilities prevented the age response study described above.

To test the hypothesis concerning cross-species sensitivity, we performed simultaneous exposures of largemouth bass and FHM to aqueous estrogens. Both species of fish were exposed to either a poultry litter extract or an estrogenic positive control for 14 days. Since Vtg has previously been induced in FHM in similar experimental settings, we predicted that we would be able to duplicate these findings and therefore validate our experimental exposure (Yonkos et al., 2010). In addition, this component of our research enabled us to compare two species, one sensitive to estrogen-induced feminization in the laboratory (FHM), and one with intersex in wild populations (largemouth bass).

2. LITERATURE REVIEW

2.1. Largemouth Bass (*Micropterus salmoides*)

Micropterus salmoides, or largemouth bass, are a type of ray-finned fish named for their large lower jaw, which extends past the back edge of their eyes (Figure 2). They are found throughout Maryland in large, slow moving rivers, soft-bottomed streams, and lakes of various sizes. Largemouth bass are aggressive carnivores, making them a highly recognized predator that regularly feeds on minnows, sunfish, gizzard shad, insects, frogs, and occasionally snakes (MD DNR, 2007). Largemouth bass are ecologically significant keystone predators as they function to maintain organization and diversity in their ecological communities (Mills et al., 1993). Changes in populations of upper and mid trophic level fish have the potential to considerably alter energy flow and biomass at lower trophic levels (Link, 2002). Largemouth bass occupy an upper trophic level, as

they are carnivorous predators, and thus any deviation from their typical population has cascading consequences for the entire aquatic ecosystem (Schindler et al., 1997).



Figure 2. Mature largemouth bass (*Micropterus salmoides*)

Reproductively, largemouth bass are gonochoristic. After fertilization, cells developing around regions of granular cytoplasm in the egg remain undifferentiated during early developmental events. These cells eventually develop into various somatic or primordial germ cells, which later differentiate to create the ovaries and testes (Devlin & Nagahama, 2002). This granular cytoplasm is observable in largemouth bass embryos 32 hours post-fertilization. During primordial germ cell development, otherwise known as the indifferent period, males and females are indistinguishable from one another. This period spans from fertilization to approximately 30 days post-hatch, when distinguishable gonads can be observed. By 37 hours post-fertilization, the first signs of germ cell migration are observable. By 3 to 5 days post-hatch, germ cells have reached the site of

the future gonad. At 10 to 12 days post-hatch, the alignment of the cells is spaced, thus displaying a bead-like appearance of the gonad. The germ cell aggregation is covered by a thin epithelium, and longitudinally aligned in anticipation of the columnar shape of the mature gonad. The germ cells continue to grow in number and size. By 25 to 30 days post-hatch, fry (young fish) are about 20 millimeters in length, spaces in the gonad are filled, blood supply to the gonad is established, and fry display the major characteristics of the largemouth bass species (Johnston, 1951).

Until 30 days of age, the germ cells remain in the indifferent period. Sexual determination of the gonad into ovaries or testes is observable by the time the fry is 3 centimeters in length, which is about 4 weeks of age (Johnston, 1951). Sexual differentiation is driven by estradiol, the maturation hormone, 17α , 20β -DP, and gonadotropins released from the pituitary gland, which differentiate germ cells into either spermatogonia or oogonia (Devlin & Nagahama, 2002). Oogonia formation is observable sooner than spermatogonia, which has also been observed in most other fish species (Dietrich & Krieger, 2009). By the time the fry is 3 centimeters in length, oocyte formation can be observed. However, spermatogonia formation is not observable until 4.5 centimeters, about 42 days post hatch. Oocytes in 3.5-centimeter long fry show the beginnings of primary yolk formation. By 6 to 8 centimeters in length, 4 months post hatch, ovaries reveal secondary yolk formation in their larger oocytes. As sexual differentiation is occurring, the gonads are developing into their characteristic, mature structure. In testes, definite tubular organization is reached once the fry is 5 to 6.5 centimeters in length, 3 months post hatch. Ovaries of fry 6 to 8 centimeters in length appear as swollen, elongated structures (Johnston, 1951).

Although the gonads have differentiated into their masculine or feminine phenotypes, reproductive competency does not occur until maturity. Maturation times in fish are heavily dependent on temperature. Since Maryland has prominent seasonal changes, the maturation time for Chesapeake Bay largemouth bass takes approximately 2 years. Once matured, the fish can reproduce and spawn. Largemouth bass spawning periods can occur as early as March and as late as June, depending on water temperatures for the year. The male fish are responsible for building nests and guarding the eggs until they hatch (MD DNR, 2007).

2.2. Sex Determination in Largemouth Bass

Unlike humans, fishes are extremely plastic in their sexual differentiation. Male humans contain the SRY gene, which genotypically makes them males. Most fishes do not have rigidly defined chromosomal sex. In fact, it has been hypothesized that there exists multiple sex determining genes on multiple chromosomes that interact and compete with the environmental and hormonal cues to determine the sex of a fish (Fitzpatrick et al., 1994). Additionally, researchers have postulated that species-specific sex determining genes and pathways may exist in fishes (Manolakou et al., 2006). Though some debate continues, most studies agree that although the sex of most fishes is genotypically predisposed, it can phenotypically develop as the opposite sex (Fitzpatrick et al., 1994).

Environmental factors that can affect sexual differentiation in fishes include temperature and exposure to exogenous chemicals that may function as hormonal agonists or antagonists. Fish are poikilothermic and thus, their embryonic development occurs in environments where large temperature differences can occur. In general, it has

been found that at elevated temperatures, sex ratios are skewed towards males (Devlin & Nagahama, 2002). This temperature sensitivity results from the effect that temperature can have on proteins, which function within the biochemical pathways of sex determination. A particular group of proteins within the affected pathways are the estrogen and androgen receptors, which are exhibited in germ cells during early fish gonadal development. Although this sensitivity to temperature has been exhibited in other species, it has not been demonstrated in largemouth bass (Devlin & Nagahama, 2002). The variable expression of the receptors makes indifferent gonads susceptible to exogenous steroids that may mimic or inhibit natural hormones. Specifically, we are studying those steroids that exhibit estrogenic properties.

Cells within an organism's body communicate by sending and receiving hormones. Glands naturally secrete endocrine hormones which travel through the bloodstream and bind specifically to receptors in the cytoplasm of target cells and activate or deactivate chemical pathways or gene expression within the target cells (Figure 3). In largemouth bass, the hypothalamus, the pituitary, and the gonad are largely responsible for controlling reproductive behavior. The highly studied hypothalamic-pituitary-gonadal axis demonstrates how a hormone, such as estrogen, can induce the production of vitellogenin (Vtg), a glycolipoprotein found in the egg yolk of females (Bone et al., 1995). This pathway involves a positive feedback loop between estrogen and gonadotropin hormones. When estrogen levels increase, the hypothalamus secretes gonadotropin-releasing hormones that in turn signal the pituitary gland to secrete gonadotropin hormones. Gonadotropins cause the gonads to increase steroidogenesis, which produces testosterone and estrogen. These increased hormone levels activate

estrogen receptors on hepatocytes, or liver cells, which then serve as transcription factors to increase the estrogen responsive gene expression (Bone et al., 1995). One such gene is the Vtg gene which, when transcribed and translated, produces the Vtg protein.

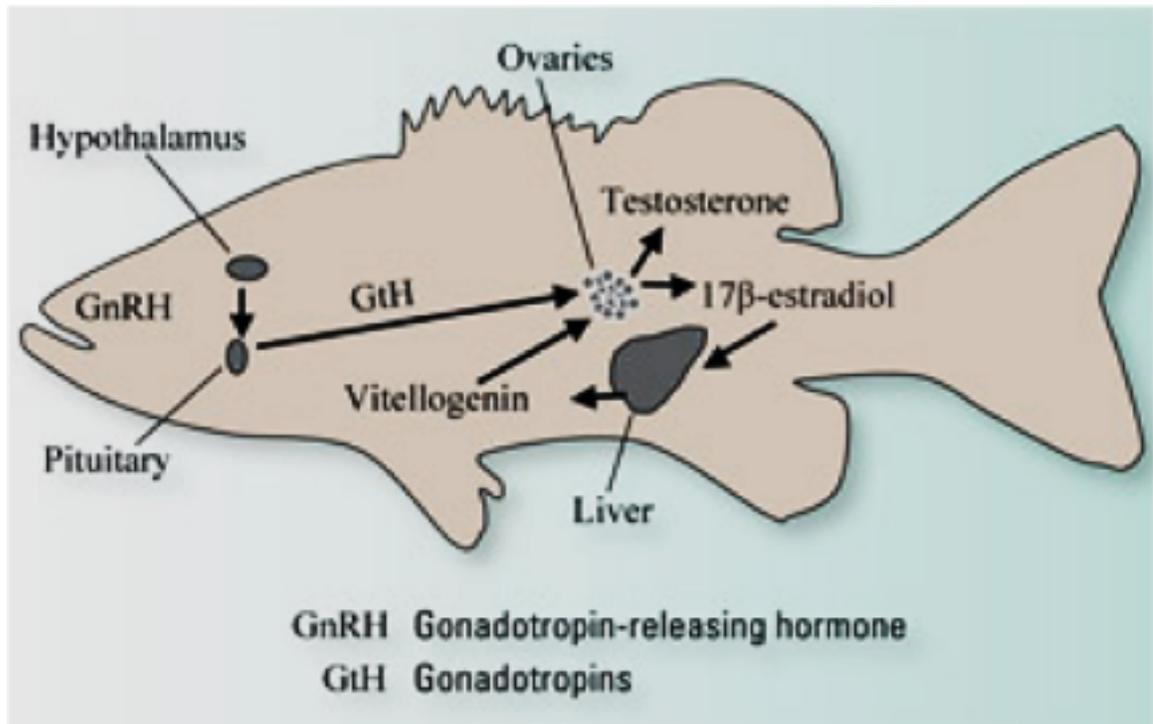


Figure 3. Generalized illustration of the hypothalamus–pituitary–gonad (HPG) axis demonstrating induction of the egg yolk lipoprotein vitellogenin in a female fish.

Endocrine disrupting compounds (EDCs) are exogenous agents that can enter an organism's body and interfere with the regulation of homeostasis and developmental processes by altering the production, metabolism and action of natural hormones (Pait & Nelson, 2003). EDCs are often shaped similarly to endogenous hormones and can interfere with hormonal pathways in two ways: they function as either agonists, mimicking the action of the body's hormones, or antagonists, preventing the original hormone from binding and carrying out its proper function (Kortenkamp et al., 2012). The natural process of Vtg production can be disturbed by the introduction of an EDC that can agonize the endogenous estrogen receptor. While females regularly

express and take up Vtg in their ovaries, males rarely express Vtg and metabolize it very slowly (Sumpter and Jobling, 1995; Panter et al., 1998; Harries et al., 1997). Males produce very little endogenous estrogen, but because they retain the estrogen receptor, EDCs are capable of inducing Vtg production in males. Thus, Vtg expression in male fish has become a valuable biomarker of exposure to estrogenic EDCs.

2.3. The Effects of EDCs

Many EDCs are human-made synthetic compounds. Alkylphenol ethoxylates (APEs), most commonly found in commercial surfactants, mimic estrogenic activity and do not degrade rapidly (Pait & Nelson, 2003). Polychlorinated biphenyls (PCBs), used in hydraulic fluids and transformers, are known for their persistent toxic effects (Kortenkamp et al., 2012). 17α -ethinylestradiol (EE2), one of the main components of oral contraceptives, is a synthetic estrogen intentionally formulated as an endocrine disruptor (Colli-Dula et al., 2014). These and numerous other chemicals are often released into natural waters through human activities or insufficient wastewater treatment at facilities that are not designed to treat trace amounts of EDCs.

Therefore, it is not surprising that observations of intersex, the expression of characteristics of both sexes, in freshwater gonochoristic fish populations have been recorded throughout the world, especially near wastewater treatment facilities (Tetreault et al., 2011). In the United Kingdom, early studies on roach (*Rutilus rutilus*) populations found a 16-100% rate of intersex at sites downstream from sewage treatment plants (Jobling et al., 1998). Intersex in Danish roach populations downstream from a sewage treatment plant were observed to be between 5-10% (Bjerregaard et al., 2006). Testicular oocyte development in German three spined stickleback (*Gasterosteus aculeatus*) and

perch (*Perca fluviatilis*) were also observed downstream from sewage treatment plants. Nearly 20% of catfish (*Clarias gariepinus*) collected from estrogen contaminated South African reservoirs were intersex (Barnhoorn et al., 2004) and approximately one out of every three spottail shiners (*Notropis hudsonius*) collected downstream from sewage plants in Montreal were found to be intersex (Aravindakshan et al., 2003). In the United States, a large scale study of fish samples collected from nine major river basins found 18% of all collected male largemouth bass (*Micropterus salmoides*) to be intersex (ranged from 9% to 91% at selected sites), making largemouth bass the second most affected fish species behind the smallmouth bass (*Micropterus dolomieu*) (Hinck et al., 2009). Most studies also found relatively low naturally occurring levels of intersex in fish collected at reference sites, thus the significantly higher rates at sites where EDCs are prevalent suggest that EDCs play a crucial role in TO formation (Blazer et al., 2007). These studies all illustrate that exposure of fish to EDCs can have direct effects on sexual development, including development of oocytes in male testes.

2.3.1. Windows of Sensitivity

Reproductive endocrine disruption can manifest in many ways, ranging from subtle and reversible increases in Vtg protein expression to testicular malformations including testicular TO development and even permanent gender reversals (Pait & Nelson, 2003) Thus, an area of significant study is in determining the ‘windows of sensitivity’ (WOS), or developmental stages at which fish are most susceptible to endocrine disruption.

Gray et al. (1998) exposed medaka (*Oryzias latipes*) to the EDC octylphenol at various ages and found that though TO development can be triggered at most life stages,

the optimal WOS was around the period of gonadal differentiation. Experiments using E2 in medaka at 15 μ g/L vs. 5 μ g/L found that higher concentrations completely altered the sex ratio, while lower concentrations could induce intersex (Koger et al., 1999). Hirai et al. (2005) also found the period of gonadal differentiation in medaka to be the most sensitive time for endocrine disruption. The authors also noted that TO begin development at the center of the testis. These studies all used concentrations of EDCs much higher than observed in nature. In FHM, ethinylestradiol (EE2) has been used to successfully induce Vtg synthesis in males at environmentally relevant concentrations (Van Aerle et al., 2002). However, Vtg synthesis was only statistically different from controls when exposures were done 20 days post hatch (dph) as opposed to the groups exposed 5-10 dph and 10-15 dph.

In rainbow trout, exposures to high concentrations of 17 α -methyltestosterone (MT) and 11 β -hydroxyandrostenedione have successfully reversed genetically male fish into functional females (Feist et al., 1995). The WOS for TO development in rainbow trout exposed to E2 has been found to be in a 24-day period between 30-54 days postfertilization (DPF) with the most sensitive period to be between 44 and 51 DPF (Krisfalusi & Nagler, 2000). In zebrafish, exposure to 17 α -ethinylestradiol (EE2) was found to cause a dose-dependent increase in Vtg production (Örn et al., 2003). Low levels of E2 (though still higher than those reported in the natural waters) have also been shown to induce TO and sex ratio changes in zebrafish when exposed prior to or during sexual differentiation (Brion et al., 2004). Maack & Segner (2004) found zebrafish were most sensitive to EE2 at comparable levels during the gonad differentiation stage.

Liney et al. (2005) exposed roach (*Rutilus rutilus*) to wastewater treatment plant effluent (WWTP) as embryos from immediately post-fertilization to 300 days post-hatch, including the time during sex differentiation. Post-spawning males were also exposed and tested in two experimental groups, adult roach which were hatched and grown to maturity in clean water, and adult roach which were hatched and grown to maturity in the wild (assuming they were exposed to some estrogen from the wild). In both life stages, Vtg induction correlated positively with measured estrogenicity in the exposure effluent (Liney et al., 2005).

Kidd et al. (2007) conducted a 7-year study at the Experimental Lakes Area in Ontario, Canada which demonstrated that chronic exposure of fathead minnows, ages 0-4 years, to a steady but low EE2 concentration (≤ 6 ng/L) resulted in endocrine disruption including feminization of males (e.g., Vtg induction and intersex, which is defined as the "presence of both male and female gonadal tissues"), and altered oogenesis in females. The study found that after only 7 weeks of estrogen addition there were higher levels of Vtg in both male and female fish. Eventually, reproduction failed within the lake population driving the species to near extinction (Kidd et al., 2007).

Collectively, the scientific literature suggests that fish species are most susceptible to endocrine disruption at a very young age, in particular the period between sexual differentiation (30 to 42 days post hatch) and gonad development (10 to 12 days post hatch). Studies into WOS in largemouth bass have not been conducted and due to the variability between fish species, conclusions about the WOS and the range of endocrine disruption possible in largemouth bass cannot be drawn without further research.

2.4. Poultry Litter as a Source of EDCs

Endocrine disruption (e.g., TO development and Vtg in male fish) has been reported in wild fish at numerous sites where there are no apparent point sources of pollution (Blazer et al., 2007; 2011; Yonkos et al., 2014). For example, in the United Kingdom, studies of roach (*Rutilus rutilus*) upstream from wastewater treatment plants found 11-45% intersex in male fish (Jobling et al., 1998). The authors suggest this indicates the existence of non-point sources of EDCs.

Steroids including estrogens and androgens are often produced through natural metabolic processes and released through excretion by domestic animals like chickens, pigs and cows. Referred to as fecal steroids, if these chemicals come into contact with other organisms, they have the potential to function as EDCs. Progesterone, E1, estradiol (E2), testosterone, and cortisol are the most common fecal steroids, all of which are highly lipophilic and only slightly soluble in water.

Poultry litter, by weight, is mainly composed of chicken manure (Shore et al., 2003). Poultry litter is primarily used as a crop fertilizer because it is nutrient rich and easily accessible to farmers. This results in an estimated yearly land application of 2.7 tons of estrogens excreted by poultry in the United States (Shore et al., 2003). When poultry litter is applied on a field, estrogen in the poultry litter binds to soil particles and is mostly concentrated in the topsoil. After heavy rainfall, it enters waterways as agricultural runoff, contributing estrone, estradiol, and estriol to aquatic systems (Peterson et. al, 2000). Once hormones, such as the potent 17 β -estradiol, enter surface waters, they will gradually adsorb to suspended particulate matter, deposit on bottom sediments or become mineralized through microbial processes (Shore et al., 2003).

Previous research indicates that some (or perhaps all) estrogens in poultry litter-amended runoff (or in laboratory litter extracts) exist as estrogen sulfate or glucuronide conjugates which are water soluble but estrogenically inactive. However, once in natural waters, these compounds are microbially deconjugated to their bioactive parent form, before being entirely degraded by the microbial community. Thus, estrogenic activity can actually increase for several days to weeks after initial poultry litter introduction before gradually decreasing to trivial levels.

Kjaer et al. (2007) conducted a study in which they were able to support the theory of estrogenic compounds such as E1 and E2 “leaching” into soils through manure. The manure containing these estrogenic compounds carries over as it moves along with the runoff into the waterways. The compounds can be found in high concentrations for as long as 3 months (Kjaer et al., 2007). Agricultural practices inadvertently incorporate these EDCs from animal excrement in an effort to enrich soil content. For example, animal manure is comprised of bedding and animal feces and urine, which contains naturally expelled estrogen and testosterone (Shore et al., 2003). The Shenandoah River watershed was studied to analyze the relationship between the percentage of animal feeding operations in watersheds and estrogenic activity. Regional surface water samples were collected and estrogenicity quantified using a BLYES assay. Results, reported as 17 β -estradiol equivalents (EEQs), exceeded 1.0 ng/L in 20% of the samples. The study suggests a positive correlation exists between the density of confined animal feeding operations (CAFOs) in a given watershed and the resulting estrogenicity within the regions receiving waters (Ciparis et al., 2012).

The effects of EDCs from agricultural runoff have been studied throughout much of the Chesapeake Bay watershed. Attention has focused principally on black bass, with particular emphasis on smallmouth bass. Blazer et al. (2007) found 73-100% of male smallmouth bass collected during the summer at various sites in the Potomac River watershed possessed TO. In initial studies, the severity and prevalence of testicular oocytes was significantly correlated to the percentage of agricultural land use of the watershed above a collection site (Blazer et al., 2014). Blazer et al. (2014) subsequently collected fish from 16 sites within three river drainages in Pennsylvania from 2007-2010. Included were smallmouth bass as well as white sucker (*Catostomus commersonii*) and redhorse sucker (*Moxostoma species*). Testicular oocytes only occurred in smallmouth bass with prevalence and severity correlating positively with the percentage of agricultural land use near that site (Blazer et al., 2014). This is in contrast to intersex observed in white suckers collected downstream from WWTPs in Boulder Creek and the South Platte River (Woodling et al., 2006). Eighty-three percent of the 60 white suckers found downstream were female, as opposed to the forty-five percent found upstream, showing a biased sex ratio towards females (Woodling et al., 2006).

Laboratory studies involving complex agricultural contaminant mixtures have been less common than those employing either single compound estrogenic exposures (e.g., E1, E2, EE2) (Schmid et al., 2002) or those employing estrogenic point source discharges (e.g., WWTP effluent) (Liney et al., 2005). However, laboratory studies using aqueous poultry litter extracts (i.e., surrogate agricultural runoff) have been shown to induce a dose-dependent increase in plasma Vtg in male FHM (Yonkos et al., 2010). In addition, exposure of larval fathead minnow to aqueous poultry litter mixtures was found

to induce feminization of male fish (Yonkos et al., 2010). Controlled exposures of fish to estrogenic contaminants in agricultural runoff are relatively uncommon, but are crucial in improving our understanding of the endocrine disrupting effect of current agronomic practices. No similar studies of PLAC exposures to black bass (smallmouth or largemouth bass) have been identified.

2.5. Fathead Minnow (*Pimephales promelas*)

Pimephales promelas, or fathead minnow (FHM), are a type of freshwater fish known to be used as feeder and baitfish (Figure 4). They are found in temperate freshwater locations throughout North America and have been known to endure and tolerate various environmental conditions. Ankley et al. (2000) reviewed reasons for using FHM as a model species for EDC testing; some reasons include how FHM are a good representative of an important family of fish, Cyprinidae, and how this particular species has already been extensively studied and tested on in relation to EDCs. The FHM are easily attainable as they are widely available and they spawn more frequently, 1-15 day spawning interval, average 3-4 days (Jensen et al., 2001). FHM, although a small fish species, are able to provide enough blood to determine plasma steroids as well as Vtg levels (Ankley et al., 2000). Therefore, we chose to compare our study with FHM as the species has been proven as a model species for EDC detection and as a species in which experimenters can induce intersex.

Studies of endocrine disruption in fish typically follow one of two models. The first includes field studies of fish populations; the second includes caged laboratory studies. Field studies typically seek to evaluate the prevalence of intersex in nature as a biomarker for environmental health, while laboratory studies often expose fish to

compounds in controlled environments in order to determine causal relationships with EDCs.



Figure 4. Mature male fathead minnow (*Pimephales promelas*) in breeding form (i.e., displaying breeding tubercles on the snout, a pronounced fatty nape pad, and dark coloration on the head and body).

Laboratory studies have shown Vtg to be inducible in FHM and mummichogs (*Fundulus heteroclitus*) from water-soluble poultry litter (Yonkos et al., 2010) and have demonstrated Vtg induction in killifish to be EDC dose-dependent (Pait & Nelson, 2003). Male FHM have been found to have prominent levels of Vtg when in waters with estrogenic compounds. Schmid et al. (2002) exposed male FHM to water conditions of either 50 ng/L EE2 or filtered water for 35 days then another 35 days of filtered water. It was observed that the EE2 exposed FHM showed an increase in Vtg protein compared to the control fish as early as day 3 of exposure; the most significant jump was observed on days 14, 21, and 28. We seek to extend this knowledge of the causative effect of EDCs to largemouth bass by studying the effects of poultry litter solutions on Vtg and testicular oocyte induction in largemouth bass compared to FHM.

3. MATERIALS AND METHODS

3.1. Experimental Overview

The overall aim of our project was to characterize the effect of estrogenic compounds on endocrine disruption in largemouth bass (LMB) and to compare these effects to those in fathead minnows (FHM). Over a two year period we conducted four 14-d laboratory assays exposing mixed gender batches of LMB of varying ages to estrogenic compounds. The final two assays also incorporated simultaneous exposures of mature male FHM (Appendix F). The schedule of fish exposures is as follows:

- *Exposure I (June 2013): 12 months post hatch (mph) LMB*
- *Exposure II (September 2013): 3 mph LMB*
- *Exposure III (November 2013): 6 mph LMB, 18 mph LMB, 9 mph male FHM*
- *Exposure IV (October 2014): 3 mph LMB, 9 mph male FHM*

The first three exposures included a poultry litter treatment with controls, while the fourth exposure involved varying concentrations of a known EDC, E2. The initial goal of the first three exposures was to determine whether age at exposure to EDCs, specifically aqueous poultry litter-associated compounds, is related to the possibility of induction of intersex and the severity of intersex development. We predicted that fish exposed at a very young age, during the indifferent period of development, would develop the most severe intersex characteristics following a period of grow-out. We exposed LMB at four different ages (approximately 3 months, 6 months, 12 months, and 18 months) to a diluent water negative control (DWC), an 17 β -estradiol positive control (PC), and a poultry litter mixture (PL).

3.1.1. Exposure I: 12 mph LMB

During Exposure I, fish were exposed in static 14-day exposures. A total of 50 LMB were randomly distributed into each of the three treatments. After the 14 day exposure, 15 randomly selected fish from each treatment were immediately sacrificed and plasma was collected for Vtg analysis. The remaining five fish were grown out under control conditions to investigate TO induction (Appendix C & D). Water samples were collected on days 0, 1, 3, 5, 7, 10, and 14 for chemical and estrogenicity analysis.

3.1.2. Exposure II: 3 mph LMB

We exposed LMB at 3 mph in a 14-day static exposure to a diluent water negative control (DWC), an 17 β -estradiol positive control (PC; nominal concentration 60 ng E2/L), and a poultry litter mixture (PL). LMB (n=150) were randomly distributed into each of the three treatments. After the 14-day exposure, 15 randomly selected fish from each treatment were immediately sacrificed and plasma was collected for Vtg analysis. The remaining fish were grown out under control conditions to investigate TO induction (Appendix C & D). Water samples were collected on days 0, 1, 3, 5, 7, 10, and 14 for chemical and estrogenicity analysis.

3.1.3. Exposure III: Multi-species Comparison of Sensitivity to Endocrine

Disruption

During Exposure III, we simultaneously exposed two age groups of LMB (6 mph (n=45) and 18 mph(n=150)) along with a cohort of mature 9-month-old male FHM (n=36). Previous studies have demonstrated that endocrine disruption can be induced in FHM at environmentally relevant estradiol concentrations. We therefore decided to compare the sensitivity to endocrine disruption of LMB to FHM. The FHM followed the

same experimental design timeline as the bass. Cohorts of the two species were exposed for 14 days to the same three treatment groups: DWC, PC, and PL. At exposure conclusion, all of the FHM and 6 mph cohort of LMB were sacrificed for plasma collection and Vtg analysis. Subsets of 15 LMB from the 18 mph cohort were also sacrificed for plasma Vtg analysis. Remaining 18 mph LMB from each treatment (n=35) were maintained under control conditions for several months before being sacrificed and having tissues prepared for TO investigation (Appendix C & D). Water samples were collected on days 0, 1, 3, 5, 7, 10, and 14 for chemical and estrogenicity analysis.

3.1.4. Exposure IV: Effect of Estradiol Concentration on Sensitivity to Endocrine Disruption

During Exposure IV, we used a flow-through system where a concentrated E2 stock solution was continuously metered into diluent water to produce a series of E2 exposure treatments. As estrogens naturally degrade over the course of several days, constantly refreshing the system with active estrogens maintained a more uniform level of estrogenicity over the two-week exposure period.

Batches of approximately 50 mature male FHM and three-month-old mixed-gender LMB were exposed for 14-d to *Low*, *Medium* and *High* E2 treatments (2 ng/mL, 9 ng/L, and 30 ng/L, respectively), and a DWC. Subsamples of eight fish of both species were removed from each treatment on days 4, 7, and 10 for immediate sacrifice and plasma collection to evaluate the effect of time on Vtg induction. We predicted that 4 days might not be sufficient time for Vtg production to detectable levels, and thus removed an additional eight fish on day 4 that were held under control conditions for an extra 3 days prior to sacrifice on day 7. On day 14, all remaining fish were sacrificed and

plasma was collected for Vtg analysis. Water samples were collected on days 0, 4, 7, 10, and 14 for analysis of estrogens and estrogenicity.

3.2. Fish Cohort Maintenance

From 2012 to 2014, we procured, exposed, and sacrificed approximately 750 mixed gender largemouth bass (LMB) and 400 mature male FHM. Collection, handling, and testing of all species adhered to protocols approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC Protocol# R-13-16).

3.2.1. Acquisition of fish

A cohort of 300 three-month-old LMB was acquired from the Joseph Manning Hatchery (Cedarville State Forest, MD) in early September of 2012. Subsets of this cohort were exposed at 12 mph (Exposure I) and 18 mph (Exposure III) in June and November of 2013, respectively. Fish numbers were as large as was practical (given facility limitations) to ensure sufficient males at sacrifice to allow statistical comparison between treatments.

A second cohort of 275 two to three-month-old LMB were acquired from Perry Minnow Farm (Windsor, VA) in September of 2013. Subsets of this cohort were exposed at 3 mph (Exposure II; n=50) and 6 mph (Exposure III; n=15) in September and November of 2013, respectively. Batches of 15 fish from all treatments (II and III) were sacrificed at the exposure conclusion for plasma collection and Vtg analysis. Cohorts of groups exposed at 3 mph (n=35) were grown to 9 mph before sacrifice (April 2014) and tissue fixation for histological examination of testes.

FHM (9 mph; n=35) were purchased from Aquatic Biosystems (Fort Collins, CO) in November of 2013 for simultaneous exposure with the 6 mph and 18 mph LMB (Exposure III described above).

A final cohort of 225 three-month-old LMB were purchased from Zetts Tri-state Fish Farm and Hatchery (Inwood, WV) in October of 2014. These fish and a second batch of eight to nine month old male FHM (n=175; Aquatic Biosystems, Fort Collins, CO) were used in Exposure IV (October 2014) to compare species sensitivity across multiple exposure concentrations and durations.

3.2.2. Largemouth Bass Housing

From September of 2012 to November of 2013, the LMB were housed at the University of Maryland - Crane Aquaculture Facility (UMCAF). UMCAF is a recirculating aquaculture facility designed to accommodate the research interests of Dr. L. Curry Woods III, who specializes in striped bass nutrition research (Appendix E). Our fish occupied ten circular fiberglass tanks (6-ft diameter; 2000 L) recirculating through a single high capacity biofilter system. Fish were fed a specifically formulated age-appropriate LMB diet (Ziegler Brothers, Inc., Gardner, PA) and observed daily. Tanks were gently cleaned as needed. Water temperature, pH, ammonia (using a LaMotte Smart3 Colorimeter Nessler's colorimeter test kit (sensitivity 0.05 ppm NH₃-N), and dissolved oxygen (DO) were monitored daily.

Following a failure of the biofiltration system at UMCAF in November of 2013, all fish being held for grow-out were transferred to an alternate holding facility within the Animal Sciences Building at the University of Maryland until sacrifice. The holding facility, an aquaculture teaching laboratory, was a smaller scale version of UMCAF. Fish

were still observed and fed daily. Water quality was also monitored daily as in a similar fashion to the UMCAF (Appendix H).

3.3. Exposure Set-up

During exposures, fish were transferred to Dr. Yonkos' Aquatic Toxicology Laboratory (ATL). The ATL is a wet laboratory in the Animal Sciences Building at the University of Maryland designed to specifically fit the research needs of both Dr. Yonkos and our Gemstone team. All exposures were carried out in this laboratory.

3.3.1. Poultry Litter and Estradiol Exposure Media Preparation

Poultry litter (accumulated material from a standard broiler operation) was sourced from a local poultry farm (Eastern Shore, MD). In preparation for the exposures, the poultry litter was manually ground and sifted in order to filter out chicken feathers and other impurities (e.g., rocks, large woody material). Poultry litter exposures (I, II, and III) were all performed in 2000 L circular fiberglass tanks. For each exposure a one kilogram batch of this purified poultry litter was then mixed into approximately 500 L of diluent water, which was aerated with air stones for 24 hours to homogenize the solution. The aeration was then turned off to allow remaining solids to settle before the supernatant was siphoned into a larger tank of diluent water. The final poultry litter mixture consisted of the soluble constituents of the original 1 kg of poultry litter diluted into 2000 L of water, resulting in a concentration of 0.5 g/L of soluble constituents in water.

The estradiol stock solutions for Exposures I-III were made by dissolving 10 mg of E2 (Sigma-Aldrich) into 100 mL of ethanol. A 1.0 mL aliquot of this stock solution was spiked into the 2000 L exposure tank for a final concentration of 50 ng/L.

Exposure IV was a flow through exposure. A concentrated stock of 350 ng/L E2 was prepared daily in dechlorinated water and held overnight in the dark. It was then metered to the three 150 L glass aquaria along with diluent water to maintain nominal exposure concentrations of 2, 9, and 30 ng/L.

3.3.2. Laboratory Set-up

During Exposures I-III, three 7-ft. diameter circular fiberglass tanks were used for the diluent water control, estradiol control, and poultry litter treatments (Figure 5). These tanks were held under static conditions and were therefore regularly maintained and cleaned. For Exposure III (Figure 6) the simultaneous exposure of three fish cohorts was achieved by suspending 60 cm lengths of perforated 6 inch PVC pipe (capped on the bottom to achieve a 10 L volume) within 200 L HDPE barrels (with stainless steel mesh windows) which were in turn placed into the 2000 L fiberglass tanks. The FHM (n=12) were placed into the PVC pipe, 6 mph LMB (n=15) were placed into the barrel and 18 mph LMB (n=50) were allowed the entire circular tanks. A submersible pump continuously transferred water (5 L/min) from the large vessel through the PVC pipe to ensure consistent exposure of media to all fish cohorts.



Figure 5. Circular 7-ft. diameter fiberglass exposure tanks used for Exposures I-III. From nearest to farthest: diluent water control, estradiol positive control, and poultry litter treatments.

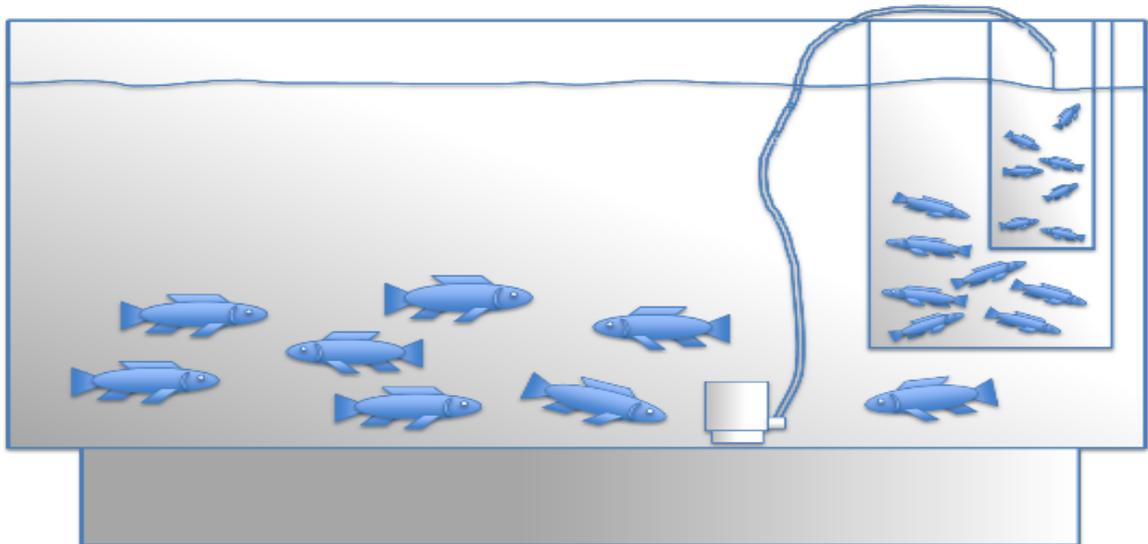


Figure 6. Exposure III set-up using a submersible pump to allow simultaneous exposure of three fish groups to equivalent contaminant concentrations: (A) 18 mph largemouth bass; (B) 6 mph largemouth bass; (C) mature male fathead minnow.

During Exposure IV, four rectangular all glass aquaria (36" x 18" x 18") with over-flow stand pipes (working volume 150 L) were used for the flow-through diluent water control (DWC) and the three estradiol treatments (Low, Medium, and High). Diluent water (dechlorinated aged aerated municipal drinking water) was metered to each of the four aquaria at approximately 350 mL/min (approximately 500 L/day) ensuring adequate water quality for fish by allowing greater than 3 volume replacements/day. The 350 ng/L E2 stock solution was metered to the Low, Medium, and High Treatment aquaria by peristaltic pump at 2 mL/min, 9 mL/min, and 30 mL/min, respectively, to achieve and maintain desired E2 concentrations. Small rectangular trays were used to separate the FHM that were simultaneously exposed with the LMB.

The exposure area was partitioned from the rest of the ATL to prevent light contamination. Lights within the exposure area were set to a 16h:8h light:dark cycle. Water quality parameters were also measured and documented every day. Water temperature ranged between 19 - 22°C throughout the exposure. The pH was between 7 - 8, but tended to be slightly basic due to the presence of ammonia from specimen waste accumulation. Ammonia levels and dissolved oxygen (DO) were also measured daily to ensure consistency and safety. DO was measured using a standard YSI meter and ammonia was measured using a LaMotte Smart3 Colorimeter with a Nessler's meter test kit (sensitivity 0.005 ppm NH₃-N).

3.4. Water Chemistry

3.4.1. Water Sample Collection & Preparation

Poultry litter (PL) and E2 positive control (PC) treatments for Exposures I-III were prepared on Day 0 and held static over the 14 day exposure periods. Because

estrogenic constituents were expected to degrade over the two week interval, water samples were collected for steroid analysis at multiple times throughout each exposure (e.g., Day 0, 1, 3, 5, 7, 10, and 14). Collection of DWC samples only occurred on days 0, 7, and 14, as the chemical environment inside this water was not expected to change over the course of the fourteen-day exposure.

On each sample day, 2 L batches of water were taken directly from each of the exposure tanks. For each filtration session, the DWC samples were filtered first, followed by the PL water, and then the PC water. This order was strictly followed to prevent residual E2 water mixing with PL water, which would have caused the estrogenicity level in PL to appear much higher than it truly was. In between each treatment type, filtration glassware was thoroughly cleaned with soap and water before nitric acid, acetone, and three distilled deionized (DDI) water rinses.

The samples were run through a two-step filtration process to ensure that no extraneous substances would be present when analyzing the samples. This was especially important for PL samples that have many undissolved matrix particles. Samples were vacuum filtered sequentially through 2.7 μ m and 0.7 μ m glass fiber filters. The filtered samples were then divided into 1 L aliquots glass bottle and a 1 L plastic bottle. The 1 L volumes were measured precisely using a 1 L volumetric flask. The glass bottles were then prepared for quantification of estrogens by liquid chromatography tandem mass spectrometry (LC MS/MS) as discussed below. The plastic bottles were used for the BLYES assay (n=1 for each tank).

3.4.1.1. Extraction Process for BLYES Estrogenicity Assessment

Samples destined for BLYES were adjusted to pH 4.0 with sulfuric acid before immediate extraction through Oasis Hydrophilic/Lipophilic balanced (HLB) Solid Phase Extraction (SPE) cartridge pre-treated with ethyl acetate, 50:50 methanol:dichloromethane, methanol, and DI water. Samples were allowed to flow through cartridges at a rate of 5-6 mL/min to completion. Cartridges were then dried for 60 minutes and either stored at -20°C (Exposure I and II) or eluted immediately (Exposure III and IV). Cartridges were eluted with 6 mL methanol and 6 mL 50:50 methanol:DCM into 15 mL glass vials which were then evaporated to dryness in a TurboVap at 35°C under nitrogen gas until dry. Once dry, samples were reconstituted with 0.5 mL of methanol before being transferred to amber vials and stored at -40°C until BLYES analysis.

3.4.1.2. Extraction Process for Estrogen Quantification by LC MS/MS

Samples destined for LC MS/MS were also adjusted to pH 4.0 with sulfuric acid prior to the extraction process. These samples were then augmented with a deuterated estrogen internal standard (supplied by Dr. Diana Aga of the University at Buffalo) to allow determination of extraction/elution efficiency and percentage recovery of unknown analytes. They were then stored in the dark at 4°C for ≤ 24 h before extraction using HLB cartridges pre-treated with methanol and DI water. Samples were then loaded and were allowed to flow through the cartridges at 5-10 mL/min. Cartridges were then dried for 60 minutes. Cartridges were eluted with two 5 mL aliquots of 98:2 methanol-ammonium hydroxide. The samples were similarly dried, reconstituted, and stored to the BLYES samples detailed above.

3.4.2. Estrogen Measurement using LC MS/MS

We partnered with Dr. Diana Aga from the University at Buffalo in order to analyze the hormonal composition of our water samples. Sample preparation (filtration, deuteration, extraction and elution) was conducted at the ATL before shipping samples priority overnight on dry ice to the Aga lab for analysis (Figure 7). Liquid chromatography tandem mass spectrometry (LC MS/MS) was then performed to simultaneously quantify E1, E2, EE2, and several estrogen conjugates using a novel method developed by Elizabeth Mullin (unpublished, University of Buffalo).



Figure 7. Agilent 6410 triple quadrupole MS equipped with an 1100 HPLC system for low level quantification of free and conjugated estrogens

3.4.3. Estrogenicity Measurement using BLYES

The *Bioluminescent Yeast Estrogen Screen* or BLYES-assay is a biological test system for the detection of estrogens in water that is based on genetically modified yeast (Alvarez et al., 2009). The BLYES assay uses the *Saccharomyces cerevisiae* strain of yeast, which is sensitive to estrogens. The yeast have been genetically modified to express both the human alpha-type estrogen receptor (hER- α) and the enzyme lacZ under the control of an estrogen responsive element (ERE) (Figure 8). If the yeast are not exposed to estrogens, they do not express any lacZ proteins. If the yeast are exposed to estrogen, however, it will bind to hER- α and initiate lacZ expression. The presence of lacZ can thus be used to determine whether the yeast have been exposed to estrogens. The expression of lacZ can be readily quantified from its enzymatic activity, generating a yellow colored product that can be measured spectrophotometrically by absorbance at 540 nm (Sanseverino et al., 2005). Because the yeast responds to estrogenic stimuli in a roughly linear fashion, total estrogenicity can be quantified in complex environmental samples.

The BLYES assay has proven to be an efficient and rapid tool for quantitation of total estrogenicity, regardless of the actual source of estrogenic activity (Sanseverino et al., 2009). Bergamasco et al. (2011) compared quantitation of estrogenic activity using BLYES with analysis of multiple estrogenic analytes by liquid chromatography-tandem mass spectrometry (LC MS/MS) and found the BLYES assay to be more accurate for detection of estrogenicity at low but environmentally relevant levels.

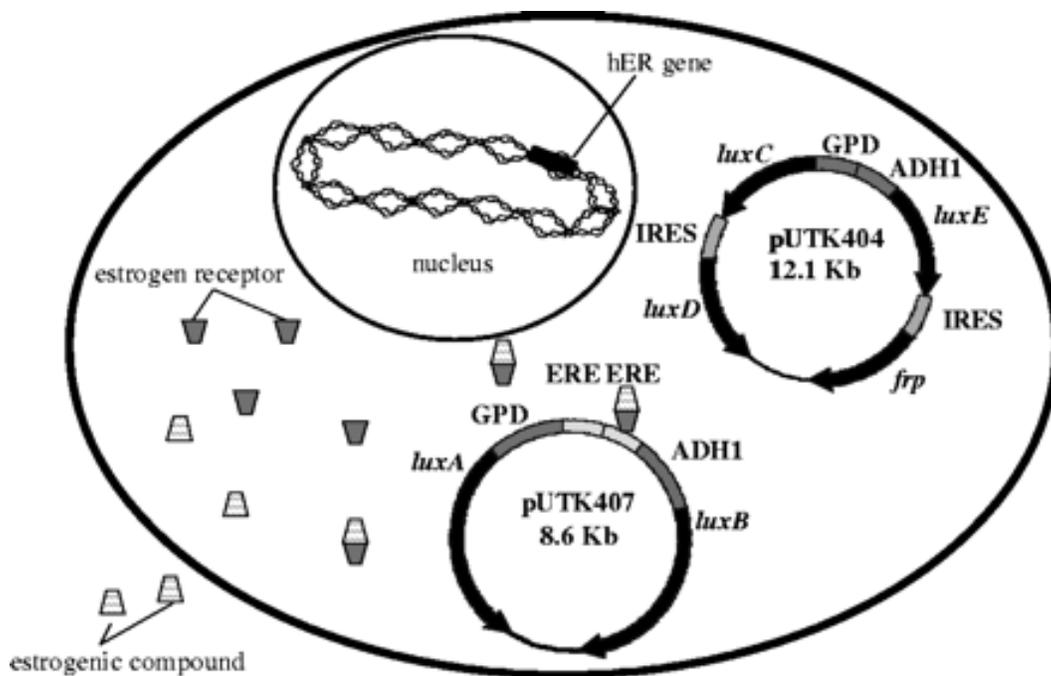


Figure 8. *Saccharomyces cerevisiae* yeast has been modified for the BLYES assay in order to express both the human alpha-type estrogen receptor (hER- α) and the enzyme lacZ under the control of an estrogen responsive element (ERE).

We cultivated a new yeast culture for use from a dormant strain of *Saccharomyces cerevisiae* (490 BioTech, Inc) (Sanseverino et al., 2005). Dormant yeast was mixed with a yeast minimal media (YMM) and maintained in a rotary incubator (30°C at 150 rpm) for 30-48 hours prior to running the assay. Dilutions of each sample extraction were prepared and added to 96-well plates in triplicates with the BLYES media. The plates were incubated for 4-6 hours on a rotary shaker and then read using a Molecular Devices SpectraMax L plate reader. A series of standards made from purified estradiol, purchased from Sigma Chemicals was run on every plate to ensure proper extrapolation of data (Appendix A).

3.5. Biological Sample Collection and Analysis

3.5.1. Fish Sacrifice and Plasma Collection

Fish were anesthetized by placing them in a 100 mg/L tricaine methanesulfonate (MS-222) water bath, proven previously to be effective (Hernandez-Divers et al., 2004; Swenson, Rosenberger & Howell, 2007; Divers et al., 2009). Bass were transferred from their respective exposure tanks to the MS-222 solution until they became immobile (approximately 5 minutes). Once anesthetized, the fish were removed from the bucket and gently patted dry. Total length (mm) and weight (0.1 g) of each fish was also recorded.

Throughout all exposures, blood plasma was collected in fish that were not destined for grow out. Fish were prepared for bleeding by removing the anal fin and descaling the caudal region. A scalpel was used to make a transverse incision through the ventral portion of the caudal region (Figure 9). The fish was then immediately lifted up and held vertically with the head elevated higher than the tail in order to allow maximum blood flow. A 70 μ L heparinized capillary tube was inserted into the incision to collect whole blood from the caudal vein. Multiple capillary tubes were used if necessary. The tubes were then plugged with a clay sealing compound, centrifuged for five minutes, and the separated plasma removed to heparinized and aprotinized cryovials, which were then snap-frozen in liquid nitrogen before storage at -40°C.

At this point, the fish was sacrificed by quickly and completely severing the brain stem near the top of the dorsal region behind the eyes, using a large heavy-duty knife. Once the fish was sacrificed, we proceeded to collect other necessary tissues from the abdominal cavity.



Figure 9. A transverse incision is made through the ventral portion of the caudal region.

3.5.2. ELISA Vitellogenin Measurement

A common physiological indicator of exposure to environmental estrogen in fish is elevated Vtg levels. Vitellogenin is a lipoprotein that acts as the energy source for developing eggs in mature female fish. As discussed previously, the expression of the Vtg gene in male fish has been used as a biomarker for the presence of endocrine disrupting compounds in the environment (Pait & Nelson, 2009). An Enzyme-linked Immunosorbent Assay (ELISA) is used to quantify Vtg levels in fish plasma. ELISA is used to detect the presence and quantity of a certain antigen in a sample that binds with a specific enzyme linked antigen. After an enzyme substrate is added to the antibody, a colored product is formed. Its intensity correlates with the amount of antigen present (Biosense Laboratories, 2005). Brion et al., (2004) utilized ELISA to measure vitellogenin in zebrafish. The assay measured Vtg induction in male zebrafish that were exposed to 17β -estradiol. Male zebrafish were exposed for 7 days to 0.1, 1.0, 10, and 100 $\mu\text{g/L}$ of E₂ which led to induction of Vtg that was dose-dependent. Lomax et al., (1998) conducted an ELISA for measurement of Vtg in English sole (*Pleuronectes vetulus*). The assay was able to detect a range of Vtg of 10–450 ng ml^{-1} (85–20% of binding) of the diluted sample.

Vitellogenin was measured in LMB and FHM plasma samples using enzyme linked immunosorbent assays (ELISA) appropriate for each species. Plasma samples were sequentially diluted (generally 1000x) before being added in duplicate to a 96-well high-binding microplate (Costar 3369), followed by incubation with a primary antibody (Mouse anti-striped bass Vtg Monoclonal antibody for LMB; Mouse anti-carp Vtg Monoclonal antibody for FHM) and Biotin-SP-conjugate Affinipure Goat anti-mouse antibody. The plates are incubated in the dark at room temperature with an added developer before the plates are read using a Spectramax M2e microplate reader at 450 nm. The absorbances recorded are indicative of Vtg concentrations within the unknown samples. Quantitation of Vtg in unknowns is achieved by comparing absorbances to the linear portion of a standard curve, generated concurrently with the plasma samples. (BioSense Laboratories, 2005) (Appendix B).

3.5.3. Fish Gonad and Tissue Collection

For mature LMB (those grown to 21 mph), the sex was recorded and the gonadosomatic index (GSI: gonad weight/total weight $\times 100$) was determined. For these fish, the gonads were removed and fixed in 10% neutral buffered formalin (NBF) for histological preparation and examination. Liver tissue was also collected in order to assess any abnormalities present in certain fish. For younger LMB grown only to 9 mph, transverse whole-body sections were taken through the abdomen and trunk regions fixed in 10%. Because gonadal tissues were generally not well-developed enough for removal, whole-body cross-sections were submitted for histological preparation so tissues could be examined in situ.

Tissue samples were collected and prepped for histological examination from the following LMB groups:

- 1a. exposed at 3 months, sacrificed immediately
- 1b. exposed at 3 months, sacrificed at 9 months
- 2a. exposed at 6 months, sacrificed immediately
- 3a. exposed at 12 months, sacrificed immediately
- 3b. exposed at 12 months, sacrificed at 21 months
- 4a. exposed at 18 months, sacrificed immediately
- 4b. exposed at 18 months, sacrificed at 21 months

This included fish from DWC, PL, and PC exposures either sacrificed immediately or grown out for variable lengths of time after exposure. Control fish from this study also provide a histological record of normal LMB gonadal maturation and development at 3 mph, 6 mph, 9, mph, 12, mph, 18, mph, and 21 mph.

To extract the gonads, an incision was made starting at the vent and going up toward the ventral fin using a scalpel. Surgical scissors were used to further open the tissue cavity. If necessary, the tissue flaps were held back and secured using surgical clamps. If liver tissue needed to be collected, forceps were used to pull the organ out slightly from the cavity. If gonads were collected, forceps were used to move the abdominal organs aside so as to access the gonads. Connective tissue was slowly removed between the gonads and the top of the abdominal cavity (Appendix I). Once fully removed, the gonads were weighed using a gram scale. Gonad tissues were initially preserved in ten percent neutral buffered formalin for a minimum of forty-eight hours to become firm and were then transferred into ethanol solution for dehydration.

3.5.4. Testicular Oocyte Detection through Histological Examination

Histology involves the microscopic examination and analysis of organismal tissues. Bateman et al. (2004) utilized 56 archived cases of intersex in European flounder (*Platichthys flesus*) in order to develop a method to evaluate the degree of testicular oocyte development when examining the histology of gonads. In this study, flounder larger than 15 cm long were sampled and gonads were extracted and fixed in 10% buffered formalin. After sections were cut at a thickness of 3 to 5 mm, longitudinally, the sections were mounted onto slides and stained with haematoxylin and eosin. They were examined using light microscopy.

Routine histological preparation of our gonad tissue was performed for thick-section light microscopy at the Department of Natural Resources Cooperative Oxford Laboratory in Oxford, Maryland. Samples were embedded in paraffin blocks, which were then thickly-sectioned at approximately $6\mu\text{m}$. Finally, samples were mounted on glass slides and stained with hematoxylin and eosin (Luna, 1968).

Tissue observation using light microscopy was performed on all slides. Each specimen, regardless of age, had three tissue samples, from the anterior, middle, and posterior regions of the gonad. Male tissues were observed more meticulously under higher magnifications than the females, as our primary goal was to identify signs of endocrine disruption in the form of testicular oocytes in males. However, level of maturity in female tissues was noted along with any irregularities.

Histological slides were blinded and randomly numbered so as to prevent observation bias. All tissues were examined under low and high magnification (10× and 40×; Olympus BH-2 binocular microscope) by a minimum of three team members and

results determined by consensus. For each tissue, specimen identification, section, and gender were recorded, along with extra notes and observations. Any occurrences of oocytes were marked.

3.6. Statistical Analysis

Quantitative data (e.g., plasma Vtg concentration) were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Where data failed the assumptions of normality or homogeneity of variance, Kruskal-Wallis ANOVA on ranks was employed. Analyses were performed using SigmaStat version 3.5 (Systat Software), with statistical significance reported at $p = 0.05$.

4. RESULTS

4.1. Survival

Our study involved exposing 750 LMB at different ages to DWC, PL, PC, and varying concentrations of E2. We additionally exposed 210 mature male FHM to these treatments simultaneously. In both exposures I and II, 150 LMB were equally distributed into three treatments. During exposure I, there was 100% survival amongst the DWC and PC treatments. There was one mortality in the PL treatment, making the survival rate for that treatment 98%. In exposure II, abnormalities were noted in some of the 3 mph LMB purchased from Perry Minnow Farm for use. Eight were therefore sent to Dr. Ana Baya of the University of Maryland Department of Veterinary Medicine to test for parasites. Results indicated numerous white "nodules" within the livers of two of the eight fish (Figure 10). These nodules represent the metacercariae stage of trematode parasites, which can cause mortality in fish if sufficiently abundant. For this reason the

entire batch of fish was treated with a four hour therapeutic praziquantel bath one week prior to starting exposure II. Even with treatment, the DWC had 8 mortalities during the two week exposure period producing an 84% survival rate. There were two deaths in the PL treatment or a 96% survival rate. In the PC treatment there was one death, or a 98% survival rate. In exposures III and IV, there was a 100% survival rate across all treatments.



Figure 10. Largemouth bass from Perry Minnow Farms, Windsor, VA with white nodules (presumably trematode metacercariae) in liver.

4.2. Estrogenicity by BLYES

The change in estrogenic activity of each treatment during exposures I-IV was measured using the BLYES assay. Resulting estrogenic activities were reported as estradiol equivalents (EEQ) in order to simplify comparison to the hormone

quantification results from the LC MS/MS analysis. Figures 11-13 depict the estrogenicity of each treatment of exposures I-III.

4.2.1. Exposure I BLYES Results

The estrogenicity of the DWC remained below 1.0 ng/L measured EEQ during exposure I (Figure 11). We observed a slight increase in concentration beginning at day 8 and peaking at 0.77 ng/L on day 14. This change could have been due to excess background noise when reading the BLYES plates or possibly due to estrogens naturally released by the LMB that culminated during this static exposure. The poultry litter treatment (PL) of exposure I similarly showed little estrogenicity, peaking at 0.80 ng/L. This is somewhat surprising given previous measured estrogen concentrations in similar aqueous poultry litter mixtures (Yonkos et al 2010). The positive estradiol control (PC) showed a large increase in estrogenicity between Day 0 and Day 1 of the exposure to a maximum of 10.95 ng/L, followed by a rapid decrease and plateau at Day 5. The increase in EEQ between Day 0 and Day 1 may reflect poor preservation of the initial sample, as we were just learning these techniques.

4.2.2. Exposure II BLYES Results

In exposure II (Figure 12), the DWC treatment again showed little estrogenicity, averaging 0.05 ng/L EEQ. The PL treatment had slightly higher estrogenicity than in exposure I, peaking at 2.27 ng/L EEQ on day 3 before steadily decreasing until the end of the experiment. Estrogenicity was not measured on Day 14 of exposure II, due to a mishandling of the sample and faulty equipment. The PC treatment again saw a peak at Day 1, at 9.8 ng/L, but with a more gradual decrease in estrogenicity through the remainder of the exposure.

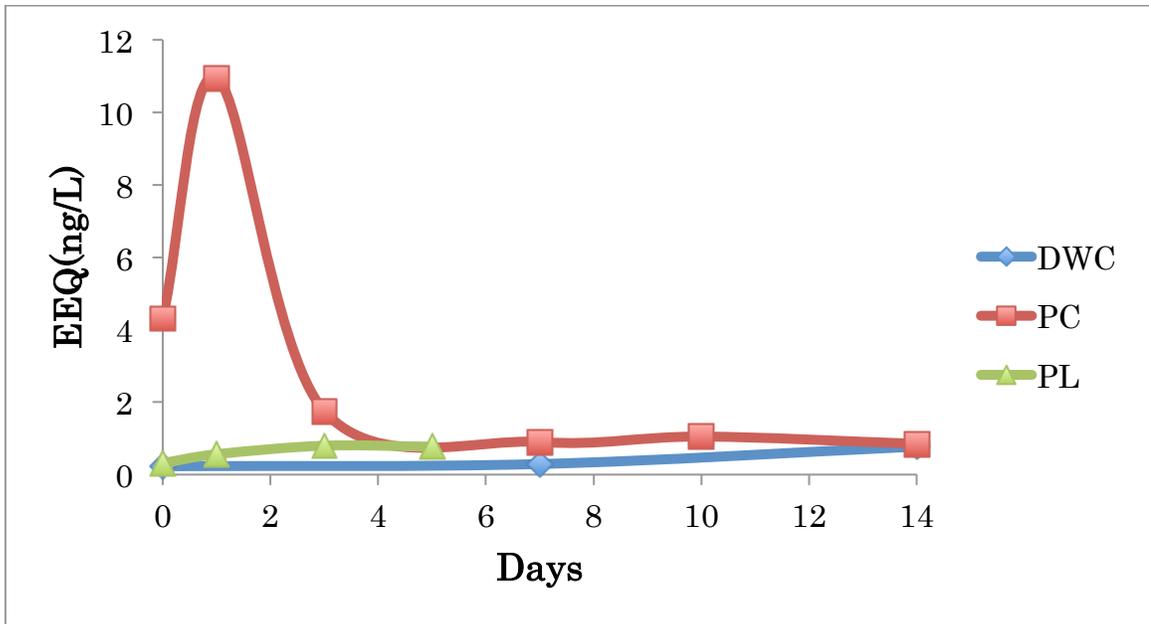


Figure 11. Estrogenicity (17β -estradiol equivalents; EEQ) of diluent water control (DWC), poultry litter (PL) and estradiol positive control (PC) treatments in exposure I as measured by BLYES assay (Sanseverino et al., 2005). Exposures were performed by preparing 2000 L treatment batches in fiberglass tanks at Day 0 and allowing them to remain static without renewal for the 14 day test period.

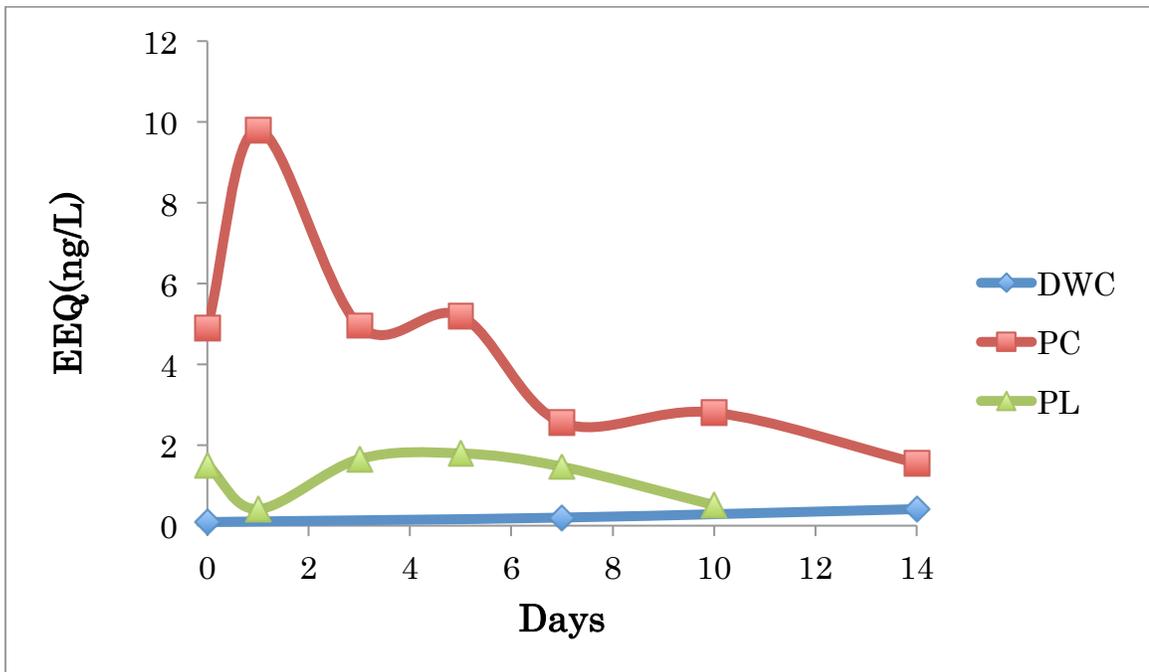


Figure 12. Estrogenicity (17β -estradiol equivalents; EEQ) of diluent water control (DWC), poultry litter (PL) and estradiol positive control (PC) treatments in exposure II as measured by BLYES assay (Sanseverino et al., 2005). Exposures were performed by preparing 2000 L treatment batches in fiberglass tanks at Day 0 and allowing them to remain static without renewal for the 14 day test period.

4.2.3. Exposure III BLYES Results

Exposure III (Figure 13) also had a DWC with nearly no detectible estrogenicity. Estrogenicity was also low in the PL treatment, peaking at 1.6 ng/L EEQ. The PC treatment was the highest at 12.9 ng/L EEQ on Day 0 of the exposure, and again rapidly decreased throughout the rest of the exposure.

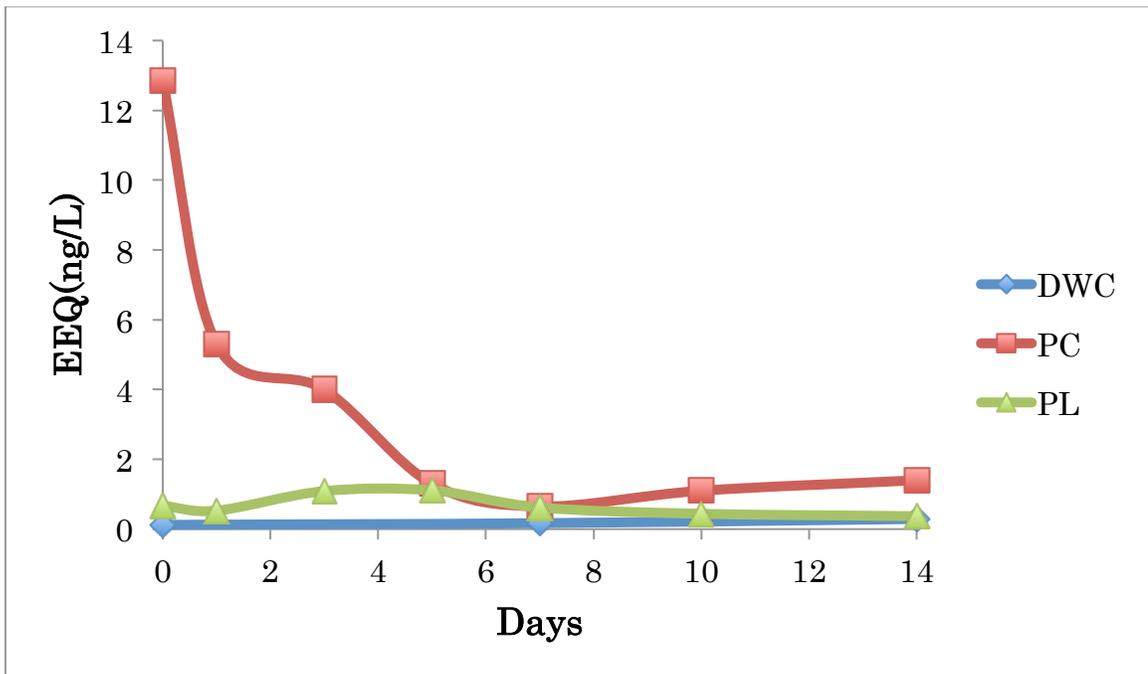


Figure 13. Estrogenicity (17β -estradiol equivalents; EEQ) of diluent water control (DWC), poultry litter (PL) and estradiol positive control (PC) treatments in Exposure III as measured by BLYES assay (Sanseverino et al., 2005). Exposures were performed by preparing 2000 L treatment batches in fiberglass tanks at Day 0 and allowing them to remain static without renewal for the 14 day test period.

4.2.4. Exposure IV BLYES Results

Exposure IV (Figure 14) employed a flow-through exposure design with diluent water and stock E2 treatments (prepared daily) metered continuously to the DWC and three E2 treatments (Low, Medium, and High) to attain target E2 concentrations. The DWC had nearly no estrogenicity as expected, averaging 0.6 ng/L. The Low E2

treatment stayed nearly constant throughout the exposure period, averaging 3.6 ng/L EEQ. The Medium E2 treatment estrogenicity was also fairly constant through the treatment, averaging 16.2 ng/L EEQ. The High E2 treatment had more variability in estrogenicity, beginning at 95 ng/L EEQ on Day 0, decreasing to 35 ng/L on Day 4, and finishing at 42 ng/L on Day 14. The average estrogenicity for the high treatment was 58.7 ng/L EEQ.

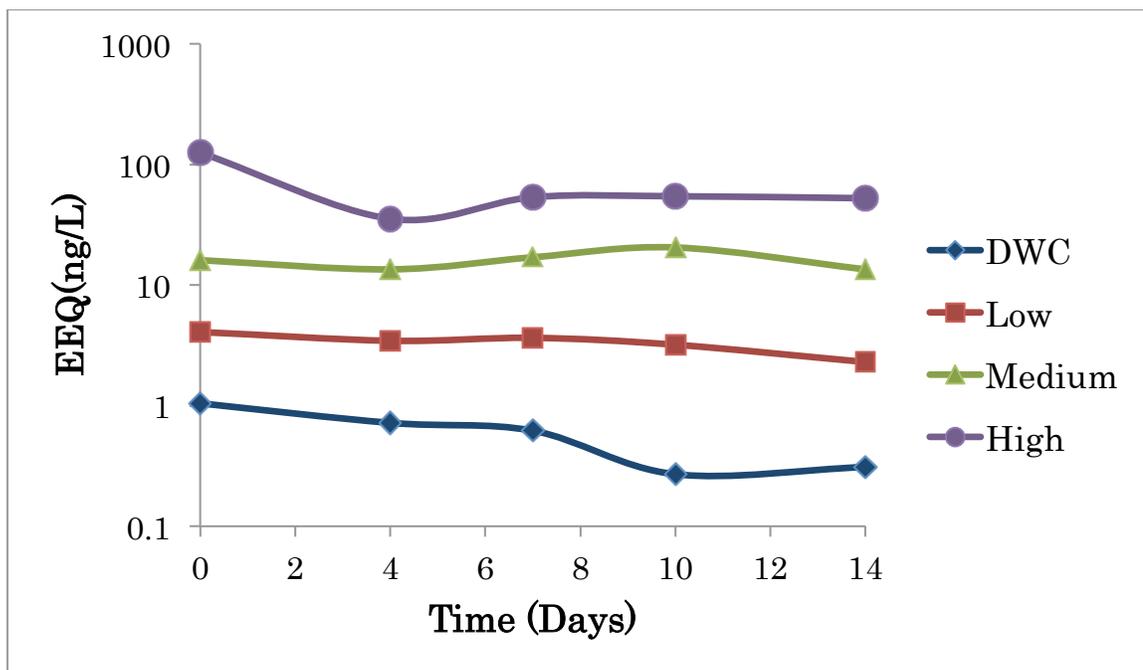


Figure 14. Estrogenicity (17β -estradiol equivalents; EEQ) of exposure IV DWC and Low, Medium and High E2 treatments during the 14 day fish exposures. This exposure employed a flow-through design with diluent water and stock E2 treatments (prepared daily) metered continuously to treatments.

4.3. Estrogen Quantification by LC MS/MS

Analysis by LC/MS/MS for various estrogen constituents was only performed on water samples from exposures III and IV. Samples from exposures I and II were deemed low priority when allocating finite resources due to concerns of poor preservation and lack of deuteration for recovery calculation. The LC MS/MS analysis performed by the Aga lab included quantification of 17β -estradiol (β E2), 17α -estradiol (α E2), and estrone

(E1), as well as the following estrogen conjugates: 17 β -estradiol-3-sulfate (E2-3S), 17 β -estrone-3-sulfate (E1-3S), 17 β -estradiol-17-sulfate (E2-17S), 17 β -estradiol-estrone-17-sulfate (E1-17S).

4.3.1. Exposure III Estrogen Quantification by LC MS/MS

In the PC treatments, β E2 and E1 (Figure 15) were detected along with the conjugates E2-3S and E1-3S which appear after approximately one week (conjugate data not shown). Since this treatment was prepared entirely from a 99% pure E2 stock, these conjugates were likely excreted from the fish. These estrogen conjugates are commonly released in livestock urine (Scherr et al., 2011). 17 β -estradiol concentration in the PC treatment began at 53.3 ng/L and gradually decreased until Day 7 where it averaged at 1.7 ng/L for the remainder of the exposure period. Figure 15 illustrated a corresponding increase in E1 coincident with the decrease in E2. Within the first 7 days of exposure III, the E2 concentration decreased from 53 ng/L while the E1 as estrone concentration increased from below detection at Day 0 to a peak of 59.9 ng/L on Day 5. The E1 concentration then rapidly decreased to 9.2 ng/L by Day 7. Figure 15 provides a comparison of the quantified hormone levels (E2 and E1) along with the estrogenicity (EEQ) of the same treatment found with the BLYES assay. The estrogenicity is a reflection of the active estrogens in the treatment, and this data should follow a similar trend to the E2 concentration, as this is the most bioactive estrogen in the treatment. The estrogenicity at Day 0 of the exposure was at 12 ng/L as compared to the 53 ng/mL 17 β -estradiol concentration. The estrogenicity followed a similar gradual decrease to the estradiol concentration, averaging at 1 ng/mL from day 7 to the end of the exposure.

In the PL treatment, E2 and E1 (Figure 16) were detected along with the conjugates estrone-3-sulphate, seen in the positive control treatment, and another conjugate E2-17S (data not shown). E2 concentration began and remained fairly low at an average of 1.18 ng/L. On the other hand, E1 concentrations were consistently higher throughout the exposure. E1 concentrations began at 8.49 ng/L on day 0, dipped down to 4.79 ng/L on day 1, and increased to a peak of 10.21 on day 5. The concentration continued to gradually decrease until the end of the exposure. The estrogenicity remained low, peaking at 1.11 ng/mL on day 5 and averaging at 0.695 ng/L. Again, the EEQ (BLYES) and E2 (LC MS/MS) trends corresponded despite the BLYES reporting EEQ concentrations significantly below the corresponding measured E2 concentrations.

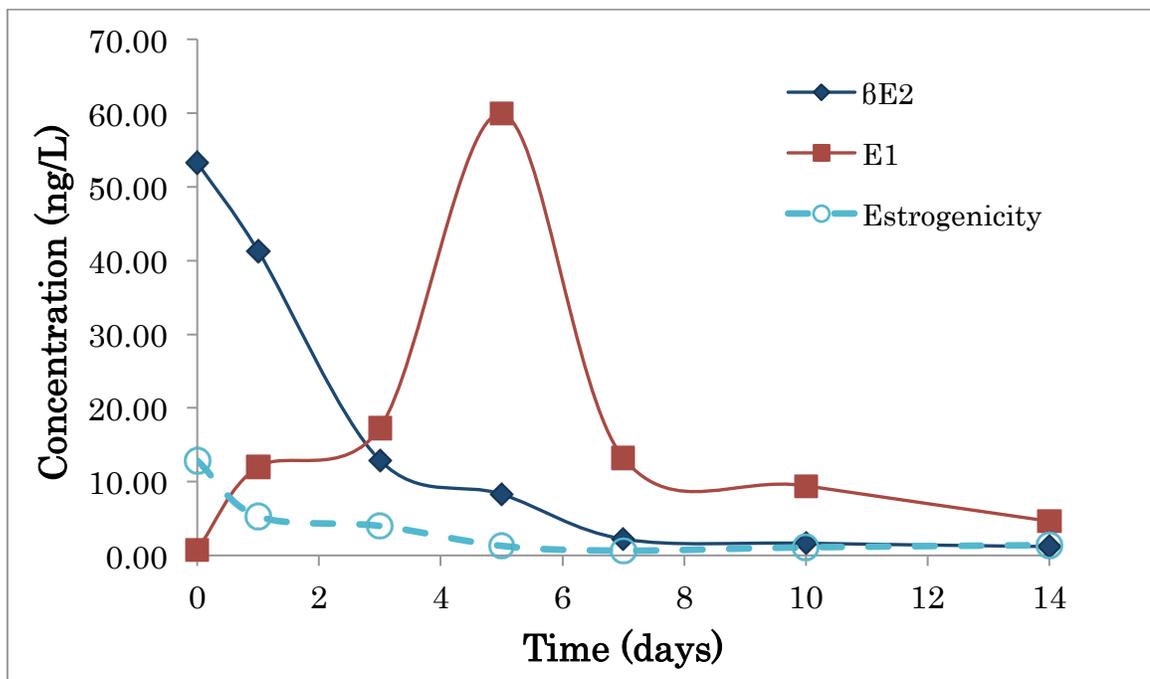


Figure 15. Measured concentrations of E2 and E1 (LC MS/MS, Dr. Aga, Univ. at Buffalo) and estrogenicity (BLYES) of the 17 β -estradiol (E2) positive control treatment from exposure III. This treatment was prepared by spiking the 2000 L PC tank with an E2 stock solution (nominal target 50 ng E2/L) at test initiation (Day 0) and allowing the treatment to degrade without renewal for the 14 day test period.

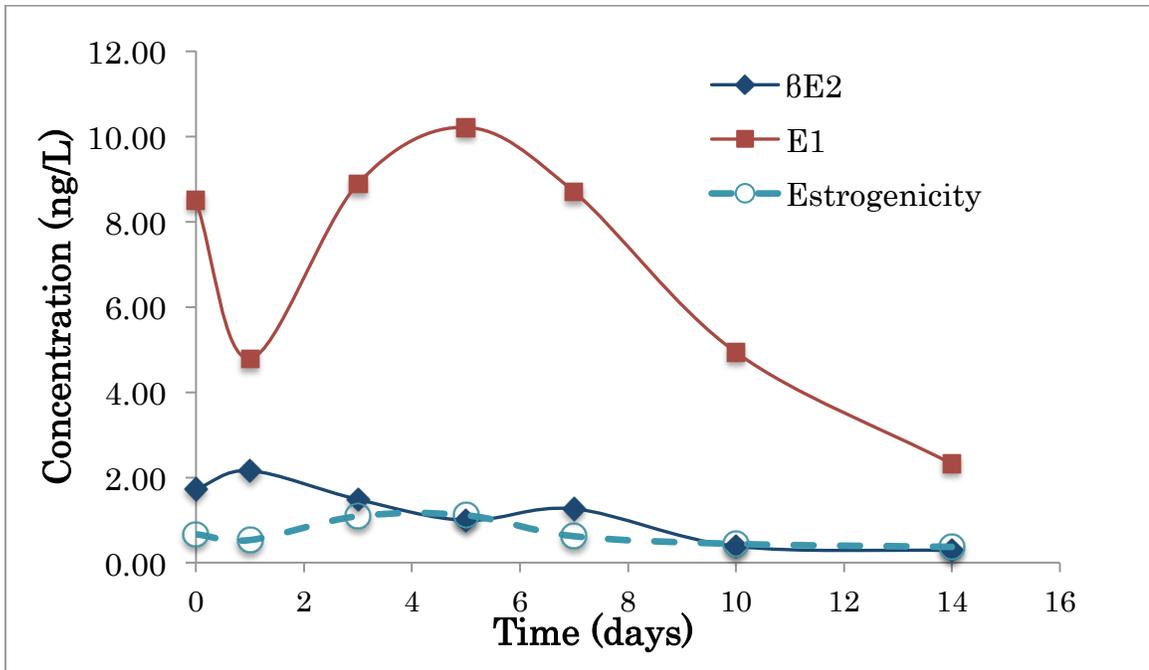


Figure 16. Measured concentrations of E2 and E1 (LC MS/MS, Dr. Aga, Univ. at Buffalo) and estrogenicity (BLYES) of the poultry litter treatment from exposure III. This treatment was prepared by mixing 1 kg of purified poultry litter into the 2000 L PL tank at test initiation (Day 0) and allowing the treatment to degrade without renewal for the 14 day test period.

4.3.2. Exposure IV Estrogen Quantification by LC MS/MS

LC MS/MS was also performed on the treatments in exposure IV with E2 and E1 concentrations quantified and compared with the estrogenicity (EEQ) results as depicted in Figure 17. Treatments largely reflect nominal E2 target concentrations of 2 ng/L (Low), 9 ng/L (Medium), and 30 ng/L (High). The control treatment showed low average concentrations of E2 (1.10 ng/L) and E1 (1.40 ng/L) and resulting estrogenicity (0.59 ng/L EEQ). The low treatment also showed low average estrogen concentrations with E2 at 4.1 ng/L, E1 at 1.20 ng/L, and estrogenicity at 3.34 ng/L. The medium treatment showed average concentrations of E2 at 12.50 ng/L, E1 at 16.17 ng/L, and estrogenicity at 0.59 ng/L. The high treatment showed a larger spike of average estrogen

concentrations with 17 β -estradiol at 29.60 ng/L, estrone at 10 ng/L, and estrogenicity at 58.7 ng/L.

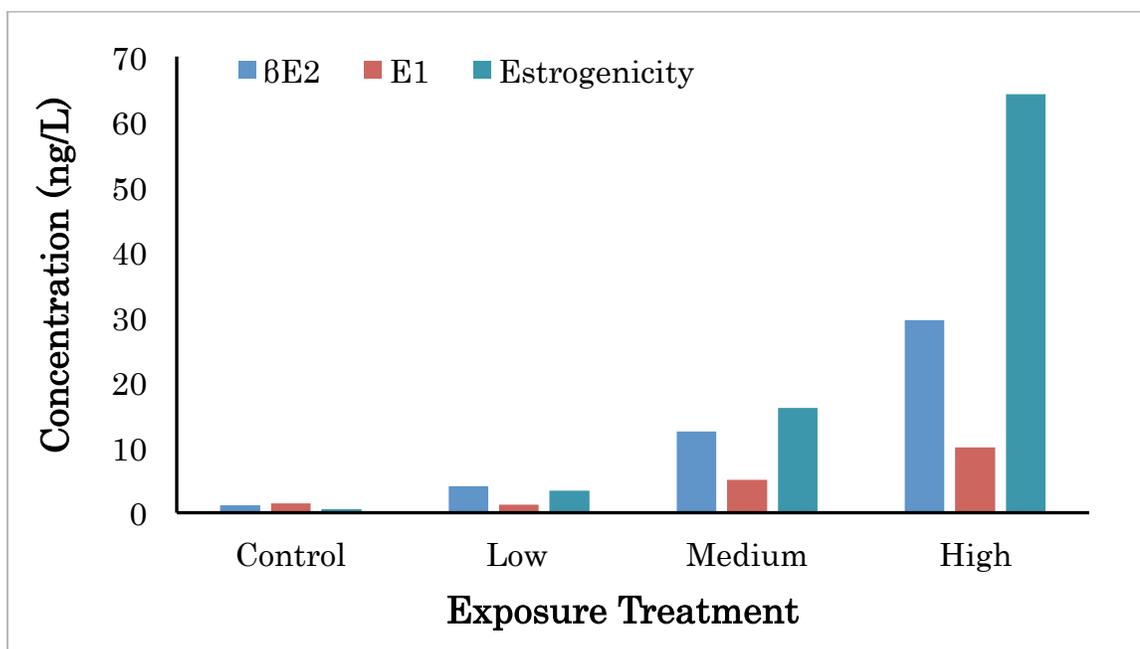


Figure 17. Measured concentrations of E2 and E1 (LC MS/MS, Dr. Aga, Univ. at Buffalo) and estrogenicity (BLYES) of diluent water control (Control), Low, Medium, and High E2 treatments in exposure IV. This exposure employed a flow-through design with diluent water and stock E2 treatments (prepared daily) metered continuously to treatments.

4.4. Plasma Vitellogenin Measured Using ELISA

Plasma samples collected from fish sacrificed immediately after exposure were analyzed for Vtg induction using ELISA. The cumulative results from Exposures I-III (all LMB treatment and age groups run against a smallmouth bass (SMB) standard) have been compiled in Figure 18. The results of the Vtg ELISA for LMB in Exposure III against a LMB standard have been compiled in Figure 19 and the results of the Vtg ELISA for LMB in Exposure IV against a LMB standard have been compiled in Figure 20. The limit of quantitation (LOQ) was determined by using our lowest standard value (0.016 μ g/mL) multiplied by the dilution factor for our samples. Those samples run against the SMB standard were at a 1:1000 dilution, making the LOQ 16 μ g/mL. Those

samples run against the LMB standard were at a 1:500 dilution, making the LOQ 8 $\mu\text{g}/\text{mL}$. For statistical and graphical purposes plasma concentrations below the LOQ were assigned a value of $\frac{1}{2}$ the LOQ for calculate treatment mean Vtg concentrations.

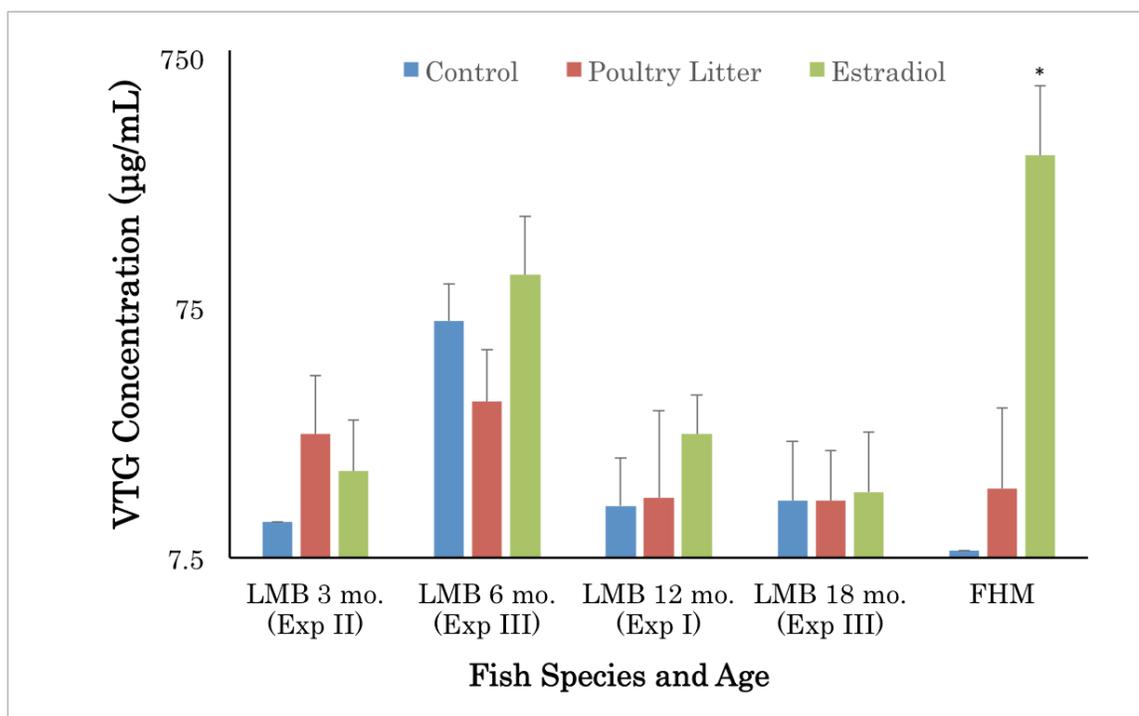


Figure 18. Mean plasma Vtg concentrations for varying ages (mph-months post hatch) of largemouth bass (LMB) and fathead minnow (FHM) exposed to control, poultry litter, and estradiol treatments in Exposures I – III (LMB plasma samples run using a smallmouth bass standard; asterisk indicates statistical significance at $p = 0.05$ as compared to control within species and age groups)

4.4.1. Exposure I Vtg Results

The Vtg results from this exposure are represented under the name LMB 12 mph in Figure 18. These plasma samples were run against a SMB standard. The average concentrations for the controls and poultry litter treatments were $12.1 \mu\text{g}/\text{mL}$ and $13 \mu\text{g}/\text{mL}$, respectively, which is under the detection limit. The estradiol exposure had an average Vtg concentration of $23.3 \mu\text{g}/\text{mL}$. All results were highly variable and did not indicate a statistically significant treatment effect.

4.4.2. Exposure II Vtg Results

The Vtg results of this exposure are represented under the name LMB 3 mph in Figure 18. These plasma samples were run against a SMB Vtg standard. We found the average Vtg concentration for controls to be 10.4 µg/mL which is below the detection limit. The poultry litter exposed fish had an average Vtg concentration of 23.4 µg/mL while the estradiol exposed fish had an average Vtg concentration of 16.6 µg/mL. Slight elevation in average PL and PC Vtg concentrations may indicate a modest induction in plasma Vtg within 3 mph fish, but high variability preclude detection of statistical significance ($P=0.05$). Even if levels prove to indicate Vtg induction, the magnitude is very low and likely not of biological significance.

4.4.3. Exposure III Vtg Results

The Vtg results of this exposure are represented under the name LMB 6 mph, LMB 18 mph and FHM in Figure 18. The LMB samples were all run against the SMB Vtg standard and the FHM samples were ran against a FHM Vtg standard.

For the 6 mph samples, we had detectable moderate amounts of Vtg in all three experimental groups, suggesting the potential that the fish had elevated Vtg at the onset of the exposure. Nevertheless, there was no evidence of a treatment related induction of Vtg compared to controls. In the 18 mph samples, we detected mean Vtg concentrations of 12 - 14 µg/mL in the Control, PL and PC groups with no evidence of treatment effect. In the FHM Exposure groups, plasma Vtg was entirely below detection in Control fish, moderate plasma Vtg was measured in two of eight PL fish (treatment mean 14.2

$\mu\text{g/mL}$ with non-detects = $8 \mu\text{g/mL}$), and statistically significant Vtg induction in the PC group (mean $304 \mu\text{g/mL}$).

The Vtg results of the LMB samples from exposure III, run against a LMB Vtg standard, are presented in Figure 19. Using this standard, measured plasma Vtg concentrations for 6 mph and 18 mph LMB were substantially higher than with the SMB standard (Figure 18), but again, no statistically significant differences were noted between Control, PL and PC treatments.

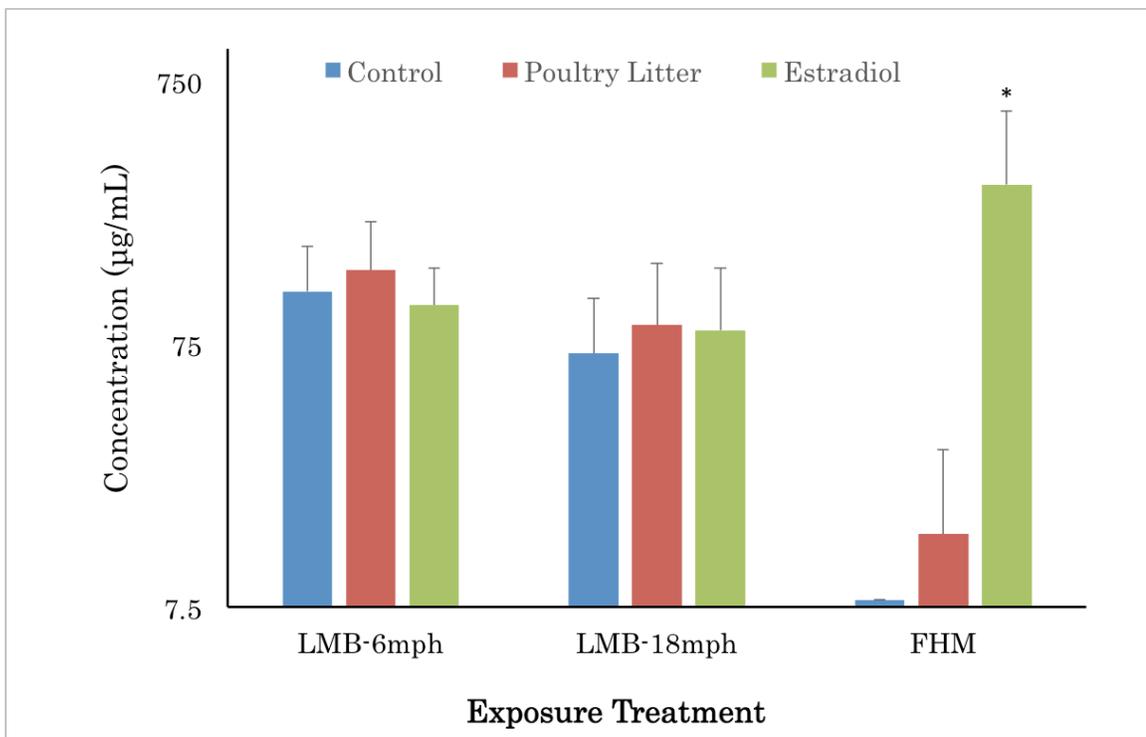


Figure 19. Mean plasma Vtg concentrations for varying ages (mph-months post hatch) of largemouth bass (LMB) and fathead minnow (FHM) exposed to control, poultry litter, and estradiol treatments in Exposure III. These samples were run using a LMB standard. Asterisk indicates statistical significance at $p = 0.05$ as compared to control within species and age groups.

4.4.4. Exposure IV Vtg Results

Results of LMB plasma samples for Exposure IV (run against a LMB Vtg standard) are presented by exposure type in Figure 20. The control Vtg concentrations

were below detection for all time points other than at Day 4 (the first examination point). Vitellogenin was generally low and variable in the three estradiol exposure treatments (Low, Medium, and High) at all sample times (mean plasma Vtg concentrations ranged from 10 to 26 $\mu\text{g/mL}$). No statistically significant increases in Vtg were noted and no exposure duration or dose response trends were apparent in the LMB exposures.

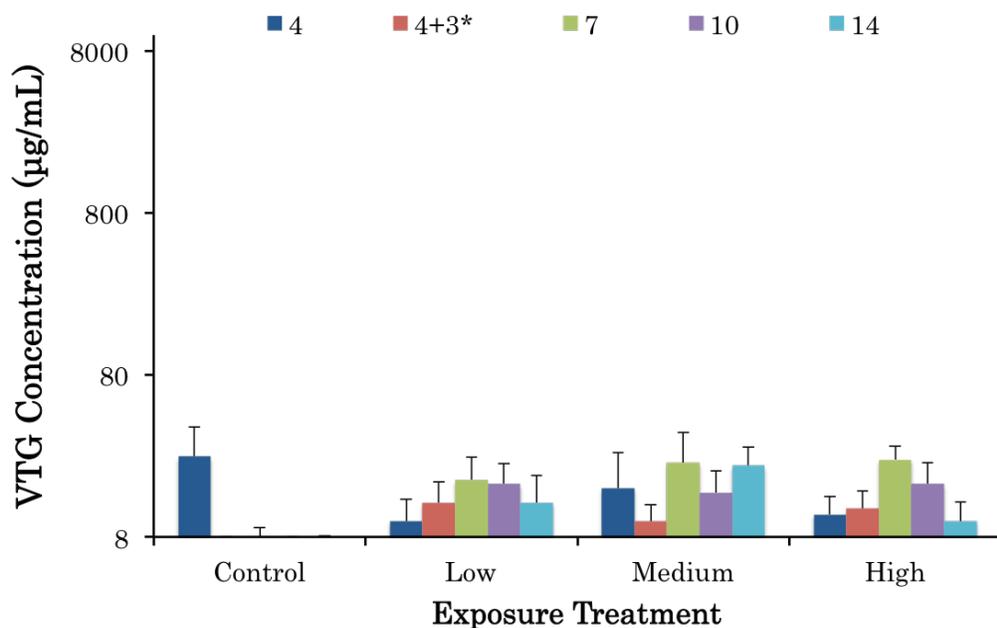


Figure 20. Mean plasma Vtg concentrations for 3 mph largemouth bass exposed to Control, Low (~ 4 ng E2/L), Medium (~ 12.5 ng E2/L), and High (~ 29 ng E2/L) estrogen treatments for 4, 7, 10, and 14 days; (*fish were also removed after 4 days and held under control conditions before sacrifice and plasma collection for Vtg analysis). These samples were run using a LMB standard.

The FHM plasma Vtg concentrations from Exposure IV are presented in Figure 21. Plasma Vtg was minimal in Control, Low (~ 4 ng E2/L) and Medium (~ 12.5 ng E2/L) treatments at all sample times. In contrast, the High (~ 29 ng E2/L) treatment had elevated Vtg concentrations at all sample times. Surprisingly, Vtg was significantly elevated after only 4 days (mean $750 \mu\text{g/mL}$). Results do not indicate any trend related to

exposure duration, but suggest a possible sensitivity threshold between the Medium and High exposure treatments.

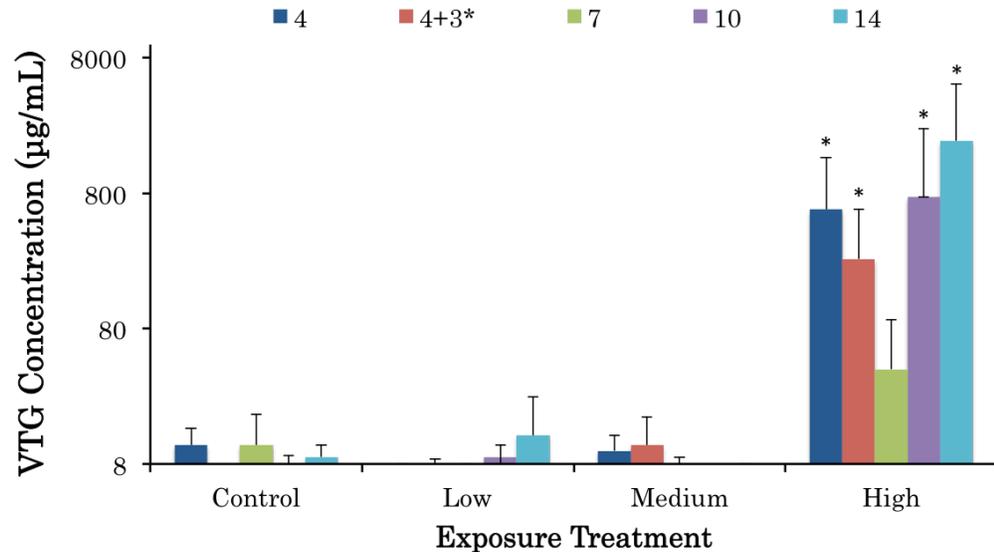


Figure 21. Mean plasma Vtg concentrations for mature male fathead minnow exposed to Control, Low (~4 ng E2/L), Medium (~12.5 ng E2/L), and High (~29 ng E2/L) estrogen treatments for 4, 7, 10, and 14 days; (*fish were also removed after 4 days and held under control conditions for 3 days before sacrifice and plasma collection for Vtg analysis on Day 7). Asterisk indicates statistical significance at $p = 0.05$ as compared to other treatment groups within day of sacrifice.

4.5. Histology

Only those fish that were held for a post-exposure grow-out period were examined histologically for the prevalence of testicular oocytes, as grow-out durations of several months were considered necessary to allow exposure-induced effects to actually manifest at the tissue level. Groups of fish examined included: 1) LMB exposed at 3 mph and sacrificed at 9 mph (n=59); 2) LMB exposed at 12 mph and sacrificed at 21 mph (n=59); and 3) LMB exposed at 18 mph and sacrificed at 21 mph (n=55). Testes from a total of 155 male LMB were examined from these three groups; 96 at 21 mph and 59 at 9 mph. Of these, nine fish were found to possess TO, all coming from batches exposed at

12 or 18 mph (which originated from the same cohort) that were then sacrificed at 21 mph (Table 1). Considered together, 3 of 31 DWC fish (9.7%), 2 of 34 PL fish (5.9%), and 4 out of 31 E2 fish (12.9%) developed TO.

Table 1: Prevalence of testicular oocytes (TO) in male largemouth bass following 14 d laboratory exposures to control, poultry litter and estrogenic positive control treatments and a subsequent grow-out period under control conditions.

	Age Exposed (mph)	Age Sacrificed (mph)	Treatment		
			Control	Poultry Litter	Estradiol
Exposure I	12	21	3/13 <i>(23%)</i>	1/16 <i>(6.25%)</i>	1/12 <i>(8.30%)</i>
Exposure II	3	9	0/21 <i>(0%)</i>	0/20 <i>(0%)</i>	0/18 <i>(0%)</i>
Exposure III	18	21	0/18 <i>(0%)</i>	1/18 <i>(5.50%)</i>	3/19 <i>(15.80%)</i>

These results make no indication of a treatment-related induction of TO, nor do they illustrate any type of correlation between age at exposure and prevalence/severity of endocrine disruption as was originally hypothesized. Lack of an apparent treatment effect suggests that observed TO prevalence (9/96 or 9.4% in 21 mph males) may have other etiologies (e.g., crowding, hatchery practices, etc.) or may merely reflect the background occurrence of TO common to this species. It is important to remember that this negative result does not preclude the possibility that estrogenic exposures at other life stages, for longer duration, or with different post-exposure grow-out periods might produce other result including significant TO induction.

5. DISCUSSION

Field collection studies of fish throughout the United States have demonstrated that the highest prevalence of intersex nationwide occurs in black basses (Hinck et al., 2009). While several studies have suggested that bodies of water impacted by high anthropogenic activity use have increased prevalence and severity of intersex, the root cause and mechanism of such endocrine disruption is not well understood (Blazer et al. 2007; 2011; 2014). To the best of our knowledge, our study is the first to employ laboratory exposures of largemouth bass with subsequent grow-out intervals as a means of exploring the causative relationship between EDCs and intersex development. To this end, we conducted four separate exposures involving LMB at varying ages, and with varying concentrations of EDCs.

One of our initial research aims was to identify an age window of sensitivity for LMB to endocrine disruption. During Exposures I-III, we exposed four different age groups of LMB to EDCs derived from poultry litter and to a positive control of E2, as well as a negative control of diluent water. With less than two years to complete our exposures and analyses, and with limited laboratory and grow-out space, we chose to use a high poultry litter exposure concentration (limited by ammonia toxicity) and a high E2 positive control treatment (generally recognized as effective in inducing endocrine effects in conventional laboratory fish species). Thus, selected exposure concentrations were intentionally high to help ensure an induced response.

In the case of our E2 positive control treatments, analysis of estrogens (LC MS/MS) and/or estrogenicity (BLYES) demonstrated that we succeeded in exposing our LMB to concentrations of EDCs significantly greater than those generally found in the

environment. This was not particularly true for our poultry litter treatments, where measured estrogens and estrogenicity were less than frequently reported environmental concentrations (Nichols et al., 1997; Shore and Shemesh, 2003). It is important to note that for Exposures I – III, tanks were held static for 14 days with a single “pulse” dose (analogous to an agricultural run-off event), which allowed estrogens to degrade rapidly, such that maximum exposure concentrations (on the order of 50 ng E2/L in E2 positive control treatments) were only experienced for the first day before precipitous reductions in estrogenicity occurred.

BLYES analysis of the water samples collected during these exposures showed minimal estrogenicity in the DWC, a low but detectable amount in the PL, and a significant level in the PC. A slight increase in estrogenicity in DWC treatments during all three 14-d static exposures may be attributable to estrogens naturally excreted by the LMB into the exposure tank (bass were of mixed gender so approximately 50% were female). In our PC treatments, we observed a clear pattern of E2 degradation over the course of the 14-d exposure period reflecting the active microbial community decomposing the potent E2 into the modestly estrogenic estrone (E1) (Jurgens et al., 2002; Ying et al., 2002). This conversion was most notable in Exposure III where E1 arrival exactly matches E2 disappearance over the first 5 days of the exposure. Note also that estrogenic activity follows the exact trend of E2 degradation indicating the dominant role E2 plays in total estrogenicity (at least as measured by BLYES). Media from Exposures I and II were only analyzed by BLYES for estrogenicity, but not by LC MS/MS for actual estrogen analytes. Therefore, similar comparisons between observed estrogenicity and constituent estrogens were not possible. It was notable that PC

treatments from both Exposures I and II exhibited an unusual increase in estrogenicity between Day 0 and Day 1. This may have been the result of sample collection on Day 0 prior to sufficient mixing and homogenization within the 2000 L exposure tank. This would help explain why the highest E2 level in these treatments was significantly below the nominal target concentration of 50 ng E2/L.

Despite our high E2 positive control exposure concentrations (or possibly due to rapid degradation of estrogens) we saw no significant induction either in TO (following grow-out) or in plasma Vtg. Most treatment groups, including DWC, had some fish with detectable levels of plasma Vtg. As observed from the BLYES analyses, trace levels of estrogenicity did exist in the DWC and may have promoted some minimal Vtg induction in the control fish. However, since these samples were run at a 1:1000 dilution, the limit of detection was 16 $\mu\text{g/mL}$, which is higher than almost all of the average Vtg blood plasma concentrations from control treatments (the exception being 6 mph LMB where all treatments had elevated Vtg). Fish from the estradiol PC treatments generally had modestly higher mean Vtg concentrations than controls, but the differences were small and enormous variability precluded any detection of statistical significance.

Recall that during Exposure III, we simultaneously exposed the 6 mph and 18 mph LMB with mature male FHM to the DWC, PL and PC treatments. Since the FHM is a model species for this type of experiment, it responded as predicted with negligible Vtg production in the DWC, low but quantifiable production in several fish from the PL treatment and substantial Vtg induction in the PC treatment. Thus, even though the exposures were static and estrogens/estrogenicity diminished rapidly, plasma Vtg was statistically significantly induced in the FHM PC treatment compared to the

controls. That fact that no similar Vtg induction occurred in the simultaneous LMB PC exposure is strong evidence of differences in sensitivity between the two species.

Histological examinations of testis for TO prevalence after grow-out of fish were also inconclusive. Collectively 9.4% of all male LMB grown to 21 mph (9 out of 96) presented with TO development. However, the nine fish that were intersex did not follow any pattern relevant to age or treatment type; 3 came from controls, 2 from poultry litter exposures and 4 from E2 positive controls. The individual with the highest TO severity ranking was actually from a control treatments exposed at 12 mph. The overall prevalence rate of 9.4% may actually reflect a normal background level of occurrence for this species, generally estimated at 5% - 10% (Vicki Blazer USGS; personal communication). It may also reflect consequences of dense culture during several months under hatchery conditions, or even have resulted from dense maintenance of our grow-out populations in the Crane Aquaculture Facility and Aquaculture Teaching Laboratory (Bahamonde et al., 2013).

It is not particularly surprising that none of the 59 male bass exposed at 3 mph and sacrificed at 9 mph were found to possess TO. The vast majority of these fish had immature testes with minimal or no developed spermatozoa which may suggest that they were not sufficiently mature to develop TO. Testis from fish sacrificed for plasma collected at 12 mph and 18 mph (immediately after 14-d exposures) were collected and preserved but not considered useful in assessing TO induction because the interval from exposure to oocyte manifestation was considered insufficient. These fish do, however, have potential value in narrowing the precise age of “normal” TO onset which, based on our study, is currently bounded between 9 and 21 mph. For this reason these specimens

will be prepared histologically and examined at a future time. While sample numbers are low at ~ 22 males for each age category (12 mph & 18 mph), they may still shed some light on this phenomenon.

The negative result from our histological examinations should be recognized as deriving from a very limited number of short-term exposures. In no way does this preclude the possibility that laboratory exposures at other life stages, for longer duration, or with different post-exposure/grow-out periods might produce other results in LMB including significant TO induction. In particular, it is possible that LMB sensitivity to EDCs may be greater at a much younger age than we were able to study (i.e., ≤ 6 weeks post hatch during the period of sexual differentiation which is perceived as being particularly sensitive to endocrine disruption). It is even possible that maternal transport of EDCs to maturing eggs and/or benthic exposure of pre-hatch eggs within nests contribute to future TO prevalence. Future studies should attempt to expose a wider range of ages, including very young and pre-hatch bass.

Because of the lack of Vtg induction in the 14-d static exposures (Exposures I – III where estrogens were permitted to decay rapidly), we decided to employ a flow-through exposure regime (analogous to a WWTP effluent discharge) where EDC concentrations would be maintained throughout the entire 14-d exposure. We introduced E2 continuously from a stock to produce LC MS/MS) confirm that the three separate concentrations of active estrogens remained relatively constant throughout the 14-d exposure. Interestingly, estrogenicity as high as 1.0 ng EEQ/L was encountered in the DWC (indicated by BLYES). This also coincided with low level detection by LC MS/MS of ethynylestradiol (1.0 – 2.0 ng EE2/L) in all treatments including the DWC

(data not shown). The only plausible source of EE2 to the system is from the city drinking water which only received dechlorination, temperature adjustment and aeration before being employed in the exposures. This is an area of concern as it has the potential to compromise future endocrine studies. More important, it is probably wise to determine if regional drinking water actually has physiologically significant levels of EDCs.

Despite maintaining consistent 14-d E2 concentrations including a High treatment averaging 30 ng/L, results of LMB Vtg analysis were again inconclusive. All Low, Medium and High treatments had some fish with detectable Vtg (as did some of the control fish). However, differences between treatments were not statistically significant at any of the sampled exposure intervals (i.e., 4 d, 7 d, 10 d, and 14 d). Recall that fathead minnow were simultaneously exposed with the LMB and that this species produced elevated Vtg in the High treatment at all of the sampled exposure intervals. This further suggests that LMB are less than ideal as a model species for laboratory experimentation into endocrine effects the need for a particularly long exposure duration before response induction.

Finally, while we were unable to cause significant TO or Vtg induction within the LMB in our exposures, these results should not be interpreted as proof that EDCs do not induce intersex in field settings. One potential explanation is the cumulative effect of low-level EDC exposure over time. For example, in natural waters bass may be exposed to poultry litter associated estrogens in seasonal cycles of several months over the course of several years. Likewise, WWTP effluents can introduce low but continuous doses of EDCs to receiving waters. Due to time constraints, our laboratory exposure model only

included single 14d exposures at specific ages. We were unable to evaluate the cumulative effect of repeated and/or long-term exposures to EDCs that more closely resemble natural conditions. Nevertheless, our results suggest that LMB may not be the best model species for detection of endocrine detection in a time-constrained laboratory setting.

BIBLIOGRAPHY

- Agriculture, U.S.D.A. (2002). Census of Agriculture: U.S. Summary and State Reports (Vol. 1). Washington D.C: U.S. Government Printing Office.
- Alvarez, D. A., Cranor, W. L., Perkins, S. D., Schroeder, V. L., Iwanowicz, L. R., Clark, R. C., Mullican, J. E. (2009). Reproductive Health of Bass in the Potomac, USA, Drainage: Part 2. Seasonal Occurrence of Persistent and Emerging Organic Contaminants. *Environmental Toxicology and Chemistry*, 28(5), 1084-1095.
- Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J., & Makyen, E.A. (2000). Description and Evaluation of a Short-Term Reproduction Test with the Fathead Minnow (*Pimephales Promelas*). *Environmental Toxicology and Chemistry*, 20(6), 1276-1290.
- Aravindakshan, J., Paquet, V., Gregory, M., Dufresne, J., Fournier, M., Marcogliese, D. J., & Cyr, D. G. (2003). Consequences of xenoestrogen exposure on male reproductive function in spottail shiners (*notropis hudsonius*). *Toxicological Sciences*, 78(1), 156-165. doi: 10.1093/toxsci/kfh042.
- Bahamonde, P. A., Munkittrick, K. R., & Martyniuk, C. J. (2013). Intersex in teleost fish: are we distinguishing endocrine disruption from natural phenomena?. *General and comparative endocrinology*, 192, 25-35.
- Bai, X., Casey, F., Hakk, H., Desutter, T., Oduor, P., & Khan, E. (2013). Dissipation and transformation of 17 β -estradiol-17-sulfate in soil-water systems. *Journal of Hazardous Materials*, 260, 733-739.
- Barnhoorn, I. E. J., Bornman, M. S., Pieterse, M., & van Vuren, J. H. J. (2004). Histological evidence of intersex in feral sharptooth catfish (*Clarias gariepinus*) from an estrogen-polluted water source in Gauteng, South Africa. *Environmental Toxicology*, 19(6) 603-608. doi: 10.1002/tox.20068.
- Bateman, K. S., Stentiford, G. D., & Feist, S. W. (2004). A Ranking system for the evaluation of intersex condition in european flounder (*platichthys flesus*). *Environmental Toxicology and Chemistry*, 23(12), 2831-2836. doi: 10.1897/03-541.1.
- Bergamasco, A. M., Eldridge, M., Sanseverino, J., Sodr , Montagner, C. C., Pescara, I. C., Jardim, W. F., & Umbuzeiro, G.de A. (2011). Bioluminescent yeast estrogen assay (BLYES) as a sensitive tool to monitor surface and drinking water for estrogenicity. *Journal of Environmental Monitoring*, 13(11), 3288-93. doi: 10.1039/c1em10464k.
- Bioscience Laboratory. (2005). Fathead Minnow Vitellogenin ELISA Kit. In B. Laboratories (Ed.).

- Bjerregaard, P., Holbech, H., Kinnberg, K., Petersen, G. I., Jackson, P., Hylland, K., & Norrgren, L. (2006). Detection of endocrine disrupters: Evaluation of a fish sexual development test (FSDT). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *144*(1), 57-66.
- Blazer, V., Iwanowicz, L., Henderson, H., Mazik, P., Jenkins, J. A., Alvarez, D. A., & Young, J. A. (2011). Reproductive endocrine disruption in smallmouth bass (*Micropterus dolomieu*) in the Potomac River basin: spatial and temporal comparisons of biological effects. *Environmental Monitoring and Assessment*, *2012*(184), 25.
- Blazer, V. S., Iwanowicz, L. R., Iwanowicz, D. D., Smith, D. R., Young, J. A., Hedrick, J. D., . . . Reeser, S. J. (2007). Intersex (testicular oocytes) in smallmouth bass from the Potomac River and selected nearby drainages. *Journal of Aquatic Animal Health*, *19*(4), 242-253. doi: 10.1577/H07-031.1.
- Blazer, V. S., Iwanowicz, D. D., Walsh, H. L., Sperry, A. J., Iwanowicz, L. R., Alvarez, D. A., Brightbill, R. A., Smith, G., Foreman, W. T., R. Manning, R. (2014). Reproductive health indicators of fishes from Pennsylvania watersheds: Association with chemicals of emerging concern. *Environmental Monitoring and Assessment*, *186*(10), 6471–6491. doi: 10.1007/s10661-014-3868-5.
- Bone, Q., Marshall, N. B., & Blaxter, J. H. S. (1995). *Biology of Fishes* (pp. 332). New York: Chapman and Hal.
- Brion, F., Tyler, C. R., Palazzi, X., Laillet, B., Porcher, J. M., Garric, J., & Flammarion, P. (2004). Impacts of 17 β -estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*). *Aquatic Toxicology*, *68*(3), 193-217. doi: 10.1016/j.aquatox.2004.01.022.
- Ciparis, S., Iwanowicz, L. R., & Voshell, J. R. (2012). Effect of watershed densities of animal feeding operations on nutrient concentrations and estrogenic activity in agricultural streams. *Science of the Total Environment*, *414*, 268–276. doi: 10.1016/j.scitotenv.2011.10.017.
- Colli-Dula, R.C., Martyniuk C.J., Kroll K.J., Prucha M.S., Kozuch M., Barber D.S., Denslow N.D. (2014). Dietary exposure of 17-alpha ethinylestradiol modulates physiological endpoints and gene signaling pathways in female largemouth bass (*Micropterus salmoides*). *Aquatic Toxicology*. 156, 148-60. doi: 10.1016/j.aquatox.2014.08.008.
- Devlin, R. H., & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, *208*(3–4), 191-364. doi: [http://dx.doi.org/10.1016/S0044-8486\(02\)00057-1](http://dx.doi.org/10.1016/S0044-8486(02)00057-1).

- Dietrich, D. R., & Krieger, H. O. (2009). *Histological Analysis of Endocrine Disruptive Effects in Small Laboratory Fish*. Hoboken, New Jersey, Canada: John Wiley & Sons, Inc.
- Dutta, S., Inamdar, S., Tso, J., Aga, D. S., & Sims, J. T. (2010). Free and conjugated estrogen exports in surface-runoff from poultry litter-amended soil. *Journal of environmental quality*, 39(5), 1688-1698.
- Feist, G., Yeoh, C.-G., Fitzpatrick, M. S., & Schreck, C. B. (1995). The production of functional sex-reversed male rainbow trout with 17α -methyltestosterone and 11β -hydroxyandrostenedione. *Aquaculture*, 131(1), 145-152. doi: 10.1016/0044-8486(94)00336-M.
- MD DNR. (2007). Fish Facts: Largemouth Bass. from <http://www.dnr.state.md.us/fisheries/fishfacts/lgmouthbass.asp>
- Fitzpatrick, M. S., Feist, G., Gale, W. L., Slater, C. H., & Schreck, C. B. (1994). Gonadal sex differentiation in fishes: Oregon cooperative fishery research unit: Oregon Cooperative Fishery Research Unit, Department of Fisheries and Wildlife, Oregon State University.
- Gray, M. A., Niimi, A. J., & Metcalfe, C. D. (1998). Factors affecting the development of testis-ova in medaka, *Oryzias latipes*, exposed to octylphenol. *Environmental Toxicology and Chemistry*, 18(8), 1835-1842. doi: 10.1002/etc.5620180834.
- Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J. P., Zaman, N. (1997). Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environmental Toxicology and Chemistry*, 16(3), 534-542. doi: 10.1002/etc.5620160320.
- Hinck, J. E., Blazer, V. S., Schmitt, C. J., Papoulias, D. M., & Tillitt, D. E. (2009). Widespread occurrence of intersex in black basses (*Micropterus* spp.) from US rivers, 1995-2004. *Aquatic Toxicology*, 95(1), 60-70. doi: 10.1016/j.aquatox.2009.08.001.
- Hirai, N., Nanba, A., Koshio, M., Kondo, T., Morita, M., & Tatarazako, N. (2005). Feminization of Japanese medaka (*Oryzias latipes*) exposed to 17β -estradiol: Formation of testis-ova and sex-transformation during early-ontogeny. *Aquatic Toxicology*, 77(86), 78-86.
- Iwanowicz, L. R., Blazer, V. S., Guy, C. P., Pinkney, A. E., Mullican, J. E., & Alvarez, D. A. (2009). Reproductive health of bass in the Potomac, USA, drainage: Part 1. Exploring the effects of proximity to wastewater treatment plant discharge. *Environmental Toxicology and Chemistry*, 28(5), 1072-1083.

- Jensen, K.M., Korte, J.J., Kahl, M.D., Pasha, M.S., & Ankley, G.T. (2000). Aspects of Basic Reproductive Biology and Endocrinology in the Fathead Minnow (*Pimephales promelas*). *Comparative Biochemistry and Physiology Part C*, 128, 127-141. doi: 10.1016/S1532-0456(00)00185-X.
- Jobling, S., Nolan, M., Tyler, C. R., Brighty, G., & Sumpter, J. P. (1998). Widespread Sexual Disruption in Wild Fish. *Environmental Science & Technology*, 32(17), 2498-2506. doi: 10.1021/es9710870.
- Johnston, P. M. (1951). The Embryonic history of the germ cells of the largemouth black bass, *micropterus salmoides salmoides* (Lacépède). *Journal of Morphology*, 88(3), 471-542. doi: 10.1002/jmor.1050880304.
- Jürgens, M. D., Holthaus, K. I., Johnson, A. C., Smith, J. J., Hetheridge, M., & Williams, R. J. (2002). The potential for estradiol and ethinylestradiol degradation in English rivers. *Environmental Toxicology and Chemistry*, 21(3), 480-488.
- Kidd, K. A., Blanchfield, P. J., Mills, K. H., Palace, V. P., Evans, R.E., Lazorchak, J.M., & Flick, R.W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences U.S.A*, 104, 8897–8901.
- Kjaer, J., Olsen, P., Bach, K., Barlebo, H. C., Ingerslev, F., Hansen, M., & Sørensen, B. H. (2007). Leaching of estrogenic hormones from manure-treated structured soils. *Environmental Science and Technology*, 41(11), 3911–3917. doi:10.1021/es0627747.
- Koger, C. S., Teh, S. J., & Hinton, D. E. (1999). Determining the sensitive developmental stages of intersex induction in medaka (*Oryzias latipes*) exposed to 17 β -estradiol or testosterone. *Marine Environmental Research*, 50(1-5), 201-206.
- Kortenkamp, A., Martin, O., Faust, M., Evans, R., McKinlay, R., Orton, F., & Rosivatz, E. (2012). State of the Art Assessment of Endocrine Disruptors: European Commission.
- Krisfalusi, M., & Nagler, J. J. (2000). Induction of gonadal intersex in genotypic male rainbow trout (*Oncorhynchus mykiss*) embryos following immersion in estradiol-17 β . *Molecular Reproduction and development*, 56(4), 495-501. doi: 10.1002/1098-2795(200008)56:4<495.
- Liney, K. E., Jobling, S., Shears, J. A., Simpson, P., & Tyler, C. R. (2005). Assessing the sensitivity of different life stages for sexual disruption in roach (*Rutilus rutilus*) exposed to effluents from wastewater treatment works. *Environmental Health Perspectives*, 113(10), 1299–1307. doi:10.1289/ehp.7921.
- Link, J. S. (April 2002). What Does Ecosystem-Based Fisheries Management Mean?. *Fisheries*, 27(4), 18-21.

- Lomax, D. P., Roubal, W. T., Moore, J. D., & Johnson, L. L. (1998). An enzyme-linked immunosorbent assay (ELISA) for measuring vitellogenin in English sole (*Pleuronectes vetulus*): Development, validation and cross-reactivity with other pleuronectids. *Comparative Biochemistry and Physiology Part B*, 121(4), 425-36. doi: 10.1016/S0305-0491(98)10125-6.
- Maack, G., & Segner, H. (2004). Life-stage-dependent sensitivity of zebrafish (*Danio rerio*) to estrogen exposure. *Comparative Biochemistry and Physiology*, 139(2004), 47-55. doi:10.1016/j.cca.2004.09.004.
- Manolakou, P., Lavranos, G., & Angelopoulou, R. (2006). Molecular patterns of sex determination in the animal kingdom: A comparative study of the biology of reproduction. *Reproductive Biology and Endocrinology*, 4(59), 59-82. doi:10.1186/1477-7827-4-59.
- Mills, L. S., Soule, M. E., & Doak, D. F. (1993). The keystone-species concept in ecology and conservation. *Bioscience*, 43(4), 219.
- Nichols, D. J., Daniel, T. C., Moore, P. A., Edwards, D. R., & Pote, D. H. (1997). Runoff of estrogen hormone 17 beta-estradiol from poultry litter applied to pasture. *Journal of Environmental Quality*, 26(4), 1002-1006.
- Örn, S., Norrgren, L., Holbech, H., Madsen, T. H., & Petersen, G. I. (2003). Gonad development and vitellogenin production in zebrafish (*Danio rerio*) exposed to ethinylestradiol and methyltestosterone. *Aquatic Toxicology*, 65(4), 97-111. doi: 10.1016/S0166-445X(03)00177-2.
- Pait, A. S., & Nelson, J. O. (2003). Vitellogenesis in male *Fundulus heteroclitus* (killifish) induced by selected estrogenic compounds. *Aquatic Toxicology*, 64(3). doi: 10.1016/s0166-445x(03)00060-2.
- Pait, A. S., & Nelson, J. O. (2009). A survey of indicators for reproductive endocrine disruption in *Fundulus heteroclitus* (killifish) at selected sites in the Chesapeake Bay. *Marine Environmental Research*, 68(4), 170-177. doi: 10.1016/j.marenvres.2009.06.006
- Panter, G. H., Thompson, R. S., & Sumpter, J. P. (1998). Adverse reproductive effects in male fathead minnows (*Pimphales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. *Aquatic Toxicology*, 42(4), 243-253.
- Peterson, E. W., Davis, R. K., & Orndorff, H. A. (2000). 17 β -Estradiol as an indicator of animal waste contamination in mantled karst aquifers. *Journal of Environmental Quality*, 29(3), 826-834. doi: 10.2134/jeq2000.00472425002900030019x.

- Sanseverino, J., Eldridge, M. L., Layton, A. C., Easter, J. P., Yarbrough, J., Schultz, T. W., & Sayler, G. S. (2009). Screening of potentially hormonally active chemicals using bioluminescent yeast bioreporters. *Toxicological Sciences*, *107*, 122–134.
- Sanseverino, J., Gupta, R. K., Layton, A. C., Patterson, S. S., Ripp, S. A., Saidak, L., & Sayler, G. S. (2005). Use of *Saccharomyces cerevisiae* BLYES expressing bacterial bioluminescence for rapid, sensitive detection of estrogenic compounds. *Applied and Environmental Microbiology*, *71*(8). doi: 10.1128/aem.71.8.4455-4460.2005.
- Scherr, F., & Sarmeh, A. (2011). Simultaneous analysis of free and sulfo-conjugated steroid estrogens in artificial urine solution and agricultural soils by high-performance liquid chromatography. *Journal of Environmental Science and Health, Part B*, *46*(8), 763-772.
- Schindler, D. E., Hodgson, J. R., & Kitchell, J. F. (1997). Density-dependent changes in individual foraging specialization of largemouth bass. *Oecologia*, *110*(4). doi: 10.1007/s004420050200.
- Schmid, T., Gonzalez-Valero, J., Rufli, H., & Dietrich, D.R. (2002). Determination of vitellogenin kinetics in male fathead minnows (*Pimephales promelas*). *Toxicology Letters*, *131*(1-2), 65-74.
- Shore, L., & Shemesh, M. (2003). Naturally produced steroid hormones and their release into the environment. *Pure and Applied Chemistry*, *75*(11-12), 1859-1971.
- Sumpter, J. P., & Jobling, S. (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environmental Health Perspective*, *103*(7), 173-178.
- Tetreault, G. R., Servos, M. R., Bennett, C. J., Shires, K., Knight, B., & McMaster, M. E. (2011). Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges. *Aquatic Toxicology*, *104*(2011), 278-290. doi:10.1016/j.aquatox.2011.05.008.
- Van Aerle, R., Tyler, C. R., Pounds, N., Hutchinson, T. H., & Maddix, S. (2002). Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow, *pimephales promelas*. *Ecotoxicology*, *11*(6), 423-434.
- Woodling, J. D., Lopez, E. M., Maldonado, T. A., Norris, D. O., & Vajda, A. M. (2006). Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. *Comparative Biochemistry and Physiology Part C*, *144* (1), 10–15.
- Ying, G. G., Kookana, R. S., & Ru, Y. J. (2002). Occurrence and fate of hormone steroids in the environment. *Environment international*, *28*(6), 545-551.

- Yonkos, L. T., Fisher, D. J., Van Veld, P. A., Kane, A. S., McGee, B. L., & Staver, K. W. (2010). Poultry Litter-Induced Endocrine Disruption in Fathead Minnow, Sheepshead Minnow, and Mummichog Laboratory Exposures. *Environmental Toxicology and Chemistry*, 29(10), 2328-2340. doi: 10.1002/etc.282.
- Yonkos, L. T., Friedel, E. A., & Fisher, D. J. (2014). Intersex (testicular oocytes) in largemouth bass (*Micropterus salmoides*) on the Delmarva Peninsula, USA. *Environmental Toxicology and Chemistry*, 33(5), 1163-9. doi: 10.1002/etc.2544.

Appendices

Appendix A: Bioluminescence Yeast Estrogen Assay

This assay involves a yeast strain that binds estrogens and then fluoresces based on the amount of estrogen bound. This assay uses the aseptic technique, so all materials must be sterile and gloves must be used.

Sterility: The interior of the fume hood and all materials must be sprayed and allowed to dry with isopropyl alcohol. Gloves should be sprayed prior to entering the hood. When working with the black 96 well plate, you should hold the cover in one hand, remove it to inject samples and then immediately recover the plate so that the plate is not exposed to contaminants in the surrounding air. Do not transfer any materials over open plates.

The standards will first be made on a clear, dilution plate (8 rows * 12 columns), and there will be two replicates for each of 12 standards, so row one and two will contain the same standards in the same order. You will add known amounts of estrogen, and then perform serial dilutions on the standards so that the concentrations are gradually decreasing across the row. Then, the standards will be transferred from rows one and two of the dilution plate to rows one and two of the actual assay plate (black). Your samples will be put into rows 3 through 8. Each sample will have 3 replicates side by side on the same row, so you can fit 4 samples (x3 replicates) on one row. You will then add the yeast and incubate it for 4 - 6 hours.

Preparing Yeast Culture

1. Add 1 ml dormant strain BLYES stored at 4°C to 100ml of fresh growth medium (about 10 ml will be needed per plate). Incubate at 30°C on a rotary shaker at 150 rpm until the culture achieves an optical density of approximately 600 nm of 0.75 to 1.0. This generally takes 30 - 48 hours.

Preparing Standards

1. Use sterile, round bottom dilution plates to prepare the 17 β -estradiol standards. The second row of the plate will contain a replicate of every column in the first row. The dilutions should be made in 5 % methanol growth media to coincide with the methanol concentration in our samples.
2. Pipette 50 mL of BLYES yeast into two 50 mL conical tubes. Centrifuge the tubes at 2000 RPM at 15 degrees C for 10 minutes. Remove from centrifuge, pour off supernatant and set aside.
3. Pipette 1 mL of methanol into another 50 mL conical tube. Add 19 mL of growth media to the conical tube to create 5% methanol growth media. Store in a solution basin for easy access.
4. Using a multichannel 100 - 1000 microliter pipette with two tips, preload standards by adding 270 microliters of 5% methanol media to the first two rows of the first column, then add 240 microliters to the third column and 200 microliters to the last column. All other columns (columns 2, 4, 5, 6, 7, 8, 9, 10, 11) receive 150 microliters of growth media. (See table) You may need to use the

micropipette multiple times in the same well if the concentration listed is higher than its capability (i.e. using a 20 - 200 microliter pipette). NOTE : during this step it is not necessary to discard tips.

Well	Add Media (µL)	Transfer (µL)	Dilution Factor	Total Estradiol content
1	270	30 (from estradiol stock of 100 ng/ml)	1:10	10 ng
2	150	150 (from well 1)	1:2	5 ng
3	240	60 (from well 2, etc)	1:5	1ng
4	150	150	1:2	500 pg
5	150	150	1:2	250 pg
6	150	150	1:2	125 pg
7	150	150	1:2	62.5 pg
8	150	150	1:2	31.25 pg
9	150	150	1:2	15.6 pg
10	150	150	1:2	7.8 pg
11	150	150	1:2	3.9 pg
12	200	0		0

5. Add 30 microliters of the estradiol stock the first column using a multichannel pipette with two tips. Mix by pipetting up and down to the FIRST stop 10x. The next step will be to dilute these wells to create the rest of the standards. NOTE: Discard pipette tips after EACH well.
6. Change tips and transfer 150 microliters from the first column to the second column. Mix 10x. Repeat with the transfer concentrations above, mixing 10x after EVERY transfer and changing tips between each duplicate set. The last well will only contain growth media. These standards will eventually be transferred to the first two rows of the final plate.

Preparing Final Assay Plate

7. Remove assay plate from packaging and make sure not touch the bottom as this is the side that is read by the plate reader.
8. Label the plate cover with a sharpie. Draw a line between the second and third rows (to indicate the first two rows are standards.) Then add lines between every

row after. Between columns 3 and 4, columns 6 and 7, and columns 9 and 10 add vertical lines to create a gridded pattern below the first two rows. Starting with row three, number each rectangle of the grid from 1 to 24 and continue that numbering onto your second plate (if you intend on running more than one at a time.) (See table below) This helps you to keep track of where you are at on your plate while loading samples. For sterility: Do not let the well cover tough the surfaces of the hood. Have it in one hand at all times.

Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9	Std 10	Std 11	Std 12
Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9	Std 10	Std 11	Std 12
s1	s1	s1	s2	s2	s2	s3	s3	s3	s4	s4	s4
s5	s5	s5	s6	s6	s6	s7	s7	s7	s8	s8	s8
s9	s9	s9	s10	s10	s10	s11	s11	s11	s12	s12	s12
s13	s13	s13	s14	s14	s14	s15	s15	s15	s16	s16	s16
s17	s17	s17	s18	s18	s18	s19	s19	s19	s20	s20	s20
s21	s21	s21	s22	s22	s22	s23	s23	s23	s24	s24	s24

9. In rows 3-8, add 95 microliters of methanol-free growth medium using a multichannel pipette with 12 tips. This keeps the sample methanol concentration consistent with the standards.
10. Add 5 microliters of sample (for a total volume of 100 microliters) to each of its designated triplicate wells, starting with a new row of pipette tips. Change tips after EVERY addition of sample (even between triplicates) and check that there is no sample remaining in the pipette before discarding it. For sample 1, add the sample to the first 3 adjacent wells in row 3, discarding tips between EACH well. Mix well by pushing to the first stop 10 times. Repeat with each sample until rows 3-8 all contain sample.
Tip: Have someone else check off each well as you add sample to it. If you lose track, count the number of pipette tips you used which will correspond exactly to where you are in the plate.
11. Using a 12 channel micropipette with 12 tips transfer 100 microliters of the standards made in the dilution plate to the the first two rows of the final assay plate. NOTE: Discard tips after EACH standard addition.
12. Using a 10 mL pipette tip, transfer 6 mL of growth media to each of the 50 mL conical tubes containing BLYES yeast from Step 2. Vortex one conical tube on 5 for 3 minutes. Transfer the entire contents of the vortexed conical tube into the second (non-vortexed) conical tube containing BLYES yeast and growth media. Then vortex this conical tube on 5 for 3 minutes. Pour the contents of this tube

into a solution basin. NOTE: both 50 mL conical tubes must be autoclaved prior to discarding in biohazard waste.

13. Add 100 microliters of BLYES media from the solution basin to each well using a 12 channel micropipette. Do not mix. NOTE: It is NOT necessary to discard tips after each transfer as long as the tips do NOT touch the liquid in the wells.
14. Cover the plate and incubate on a rotary shaker at 150 RPM and 30°C for 4-6 hours.
15. Load plate into SpectrFluorPlus plate reader and load the saved template “BLYES double read.”
16. Save raw data as an Excel final. Fit standard data into a curve.

Appendix B: Enzyme-Linked Immunosorbant Plasma Vitellogenin Assay (ELISA)

Buffers:

Dilution Buffer (PBSZ): Dissolve 1.604g trisodium phosphate, dodecahydrate (Sigma S-10010), 0.693g of monosodium phosphate, anhydrous (Sigma S-0751), 8.766g sodium chloride (S-9888) and 0.2g sodium azide (S-8032) in 900 mL 19MOhm water with gentle stirring. Once dissolved adjust to pH 7.6 with 1N HCl. (pH is already close to 7.6, so you'll only need a few drops to get there) Adjust final volume to 1L and store at 4 degrees C.

NOTE: To speed up pH adjustments in buffers **below**, 6N HCl may be used to pH adjust.

Wash Buffer (TBST): (Make 4L) Dissolve 4.844g Sigma 7-9 and 35.064g sodium chloride in 900 mL 18-MOhm water with gentle stirring. Once dissolved add 200 microliters tween-20 and adjust pH to 7.6 with 1N HCl. Adjust final volume to 4L and store at 4 degrees C.

Blocking Buffer: Dissolve 1.211g Sigma 7-9 and 8.766g sodium chloride in 900 mL 18-MOhm water with gentle stirring. Once dissolved add 10g bovine serum albumin (this takes a little time to dissolve) and 50 microliters Tween-20 and adjust pH to 7.6 with 1N HCl. Adjust final volume to 1L and store at 4 degrees C.

Carbonate Buffer: Dissolve 3.179g sodium carbonate and 0.19g magnesium chloride in 900 mL 18-MOhm water with gentle stirring. Adjust pH to 9.6 and adjust final volume to 1L. Store at 4 degrees C.

Procedure:

1. Allow 1 hour for plasma samples to defrost at room temperature
2. Label plate templates. You will use two plates for dilution, the final plate is a different plate (96-well Easy Wash High Binding, non-sterile Costar 3369) which will be read by the plate reader.
3. Once plasma samples are defrosted, centrifuge @3000rpm for ~10min

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1	STD1	STD1	s1	s5	s9	s13	s17	s21	s25	s29	s33
B	STD 2	STD 2	STD 2	s1	s5	s9	s13	s17	s21	s25	s29	s33
C	STD 3	STD 3	STD 3	s2	s6	s10	s14	s18	s22	s26	s30	s34
D	STD 4	STD 4	STD 4	s2	s6	s10	s14	s18	s22	s26	s30	s34
E	STD 5	STD 5	STD 5	s3	s7	s11	s15	s19	s23	s27	s31	s35
F	STD 6	STD 6	STD 6	s3	s7	s11	s15	s19	s23	s27	s31	s35
G	STD 7	STD 7	STD 7	s4	s8	s12	s16	s20	s24	s28	s32	s36
H	STD 8	STD 8	STD 8	s4	s8	s12	s16	s20	s24	s28	s32	s36

You will be preparing two of these dilution plates. Standards are in triplicates. Samples are in duplicates.

The first three columns of the plate are for STANDARDS. The following directions apply to the samples only, which begin on the fourth column. See the plate template for visual.

4. While plasma is in the centrifuge, prepare dilution plates. (Dilutions are based on amount of expected Vitellogenin in the plasma samples based on season and sex). Use 96-well Round Bottom Assay Plate (Costar 3360).
 - a. Dilution Buffer is PBSZ. (*Store in a solution basin for convenience*)
 - b. Dilutions
 - i. **1:100 = 2μL plasma: 198μL dilution buffer** (*first 96-well plate*)
 - ii. **1:1000 = 20μL 1:100 dilution: 180μL dilution buffer** (*second 96-well plate – final dilution*)
 - iii. **1:10000 = 2μL 1:100 dilution: 198μL dilution buffer**
 - iv. **1:100000 = 20μL 1:10000 dilution: 180μL dilution buffer**
5. Prepare dilutions by adding the required amount of dilution buffer to sample wells.
 - a. First dilution plate is 1:100 -- Add 198μL dilution buffer to each sample well.

- b. Second dilution plate is 1:1000 – Add 180 μ L dilution buffer to each sample well.
- 6. Add plasma to the first dilution plate. Be sure to mix samples by pipetting up and down (to the FIRST stop) 10x.
 - a. The first dilution plate is 1:100 -- Add 2 μ L of plasma sample to each sample well.
 - b. Be sure to pipette from the *middle* of the sample – you do not want the material from the bottom
- 7. After finishing the first dilution plate, add the required amount of the first plasma dilution to the second dilution plate.
 - a. Add 20 μ L from each well on the first dilution plate to each corresponding well on the second dilution plate.
 - b. Mix 10x times before and after transferring samples.

The following directions are for the preparation of the standards on the second dilution plate. The standards are prepared as dilutions that go from all standard to all dilution buffer. (STD2 = row 2, STD3 = row 3)

- 8. In the standard wells (first three columns) on the second dilution plate, add 100 μ L dilution buffer to **rows 2-8**. The first row (STD1) will ONLY have standard. The eighth row (STD8) will ONLY have dilution buffer.
- 9. Prepare Standard in a 25 mL centrifuge tube
 - a. Dilution of SMB Vtg = 1 μ L SMB Vtg : 2,199 μ L dilution buffer *Heather put in 2200 μ L buffer then removed 1 μ L
 - b. Dilution of LMB Vtg = 1 μ L LMB Vtg : 1,199 μ L dilution buffer
 - c. Dilution of FHM Vtg = 1 μ L FHM Vtg : 1,259 μ L dilution buffer

After creating standard, vortex to homogenize before adding it to the plate.

- 10. Add 100 μ L of diluted Vtg to STD2 wells of the dilution plate. Only insert the smallest section of the tip into the dilution buffer when adding standard. Make sure there are no remaining drips of standard on the tip. Replace tips before mixing. This will increase standard accuracy.
- 11. Prepare 2-fold dilution of standards by pipetting 100 μ L of STD2 into STD3. Again, do not insert tips into dilution buffer when *adding* STD2 to STD3 dilution buffer and just insert tip at the surface. Replace tip before mixing STD3.
- 12. Repeat this step for remaining rows except STD8, which is all dilution buffer.

Preparation of the final plate (96-well Easy Wash High Binding, non-sterile Costar 3369). DO NOT touch the bottom of the VTG plate. That is where the plate is read.

- 13. In the final plate, add 50 μ L of the diluted Vtg standard to STD1 wells. Remember STD1 is ALL standard.
- 14. Add 50 μ L of each standard and diluted sample from the second dilution plate to the corresponding wells on the final plate.
- 15. Cover plates with a plate seal and incubate in 4 degrees C refrigerator overnight. (12-18 hours)
- 16. The next morning, remove block buffer and carbonate buffer from fridge and allow 1 hour to come to room temperature.

For each of the following steps, you can use same pipette tips for the entire plate, just be careful not to touch the sample or touch the tips to the sides of the walls

17. Wash plates in plate washer 5 times. (SkanWasher 400)
18. Add 300 μ L blocking buffer to plates
19. Incubate at RT for 1 hour
20. 5 minutes prior to end of incubation, prepare primary monoclonal antibody (mAb)
[**Mouse anti-striped bass VTG Monoclonal antibody à ND-3G2**]
 - a. 5 μ L Striped bass mAb: 4995 μ L block buffer (1:1000). **Be careful when mixing the dilution because this gets foamy – swirl up and down gently.**
 - b. 5 μ L Carp mAb: 4995 μ L blocking buffer (1:1000).
21. Wash plates 5x in plate washer
22. Add 50 μ L mAb to plates
23. Incubate at RT for 1 hour
24. 5 min prior to the end of incubation, prepare secondary antibody. [**Biotin-SP-conjugate Affinipure Goat anti-mouse à Code: 115-065-003, LOT: 108597, 2.0mL**]
 - a. 5 μ L 2Ab: 4995 μ L blocking buffer (1:1000)
 - b. if 2Ab is stored in glycerol, 10 μ L 2Ab: 4990 blocking buffer
25. Wash plates 5x in plate washer
26. Add 50 μ L 2Ab to plates
27. Incubate at RT for 1 hour

28. 5 min prior to the end of incubation, prepare streptavidin-alkaline phosphatase (S-AP) [**Streptavidin Alkaline Phosphate conjugate à Ref: SA1008, LOT: 894794A**]
 - a. 1 μ L S-AP: 4999 μ L blocking buffer (1:5000)
29. Wash plates 5x in plate washer
30. Add 50 μ L S-AP to plates
31. Incubate at RT for **30 minutes**
32. Take the developer out of the freezer to thaw in the dark. Turn on plate reader (Spectramax M2e) to allow it to warm up.

33. 5 min prior to the end of incubation, prepare developer (100x 4-nitrophenyl phosphate 100mg/mL)
 - a. 50 μ L 100x N-P: 4950 μ L **carbonate buffer (1:100). Keep buffer in dark. Not as sensitive to shaking.**
34. Wash plates 5x in plate washer
35. Add 50 μ L developer to plates. **CANNOT USE SAME TIPS**
36. Incubate at RT for **30 minutes. – Put under tin foil.**

37. Read plate on microplate reader.

Appendix C: Incidence of TO in Grow-Out LMB

Sample ID	Exposure	Tissue Sample Type	Age Exposed	Age Sacrificed	Gender	Body Section	# of oocytes	Comments
C1-16	I	Gonad	12 months	21 months	M	Middle	2	
C1-22	I	Gonad	12 months	21 months	M	Middle	4	
	I	Gonad	12 months	21 months	M	Posterior	3	
C1-21	I	Gonad	12 months	21 months	M	Anterior	8	
L1-E3-27	III	Gonad	18 months	21 months	M	Anterior	1	
L1-E3-35	III	Gonad	18 months	21 months	M	Posterior	9	
L1-E3-41	III	Gonad	18 months	21 months	M	Posterior	1	
L1-P3-23	III	Gonad	18 months	21 months	M	Posterior	1	
P1-44	I	Gonad	12 months	21 months	M	Middle	4	2 in each middle gonad
E1-20	I	Gonad	12 months	21 months	M	Middle	1	
Total							34	

Appendix D: University of Maryland Crane Aquaculture Facility Layout

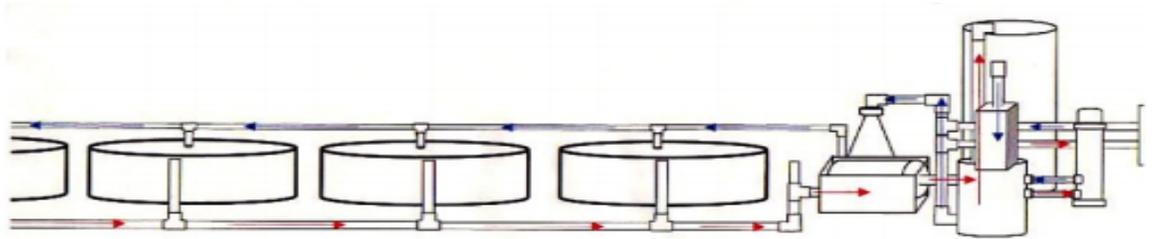
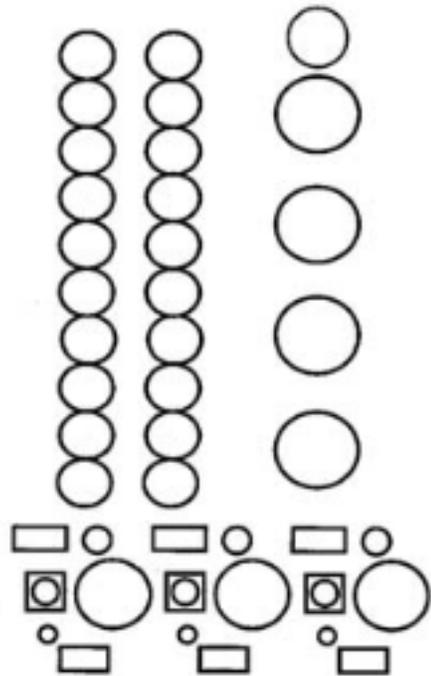


Diagram of biofilter system and tank layout



Entire UMCAF Layout

Appendix E: Overview of Exposures I-IV

Exposure Group	Exposure Number	Age/Life Stage at beginning of exposure	Exposure Treatments	Exposure Duration (days)	Age at Sacrifice
Largemouth Bass No. 1	2	3 months	DWC	14	3 months 10 months
			PL	14	3 months 10 months
			PC	14	3 months 10 months
Largemouth Bass No. 2	3	6 months	DWC	14	6 months
			PL	14	6 months
			PC	14	6 months
Largemouth Bass No. 3	1	12 months	DWC	14	12 months 21 months
			PL	14	12 months 21 months
			PC	14	12 months 21 months
Largemouth Bass No. 4	3	18 months	DWC	14	18 months 21 months
			PL	14	18 months 21 months
			PC	14	18 months 21 months
Largemouth Bass No. 5	4	3 months	DWC	4, 4+3*, 7, 10, 14	3 months
			LE	4, 4+3*, 7, 10, 14	3 months
			ME	4, 4+3*, 7, 10, 14	3 months
			HE	4, 4+3*, 7, 10, 14	3 months
Fathead Minnow No. 1	3	9 months	DWC	14	7-9 months
			PL	14	7-9 months
			PC	14	7-9 months
Fathead Minnow No. 2	4	9 months	DWC	4, 4+3*, 7, 10, 14	7-9 months
			LE	4, 4+3*, 7, 10, 14	7-9 months
			ME	4, 4+3*, 7, 10, 14	7-9 months

HE

4, 4+3*,
7, 10, 14

7-9 months

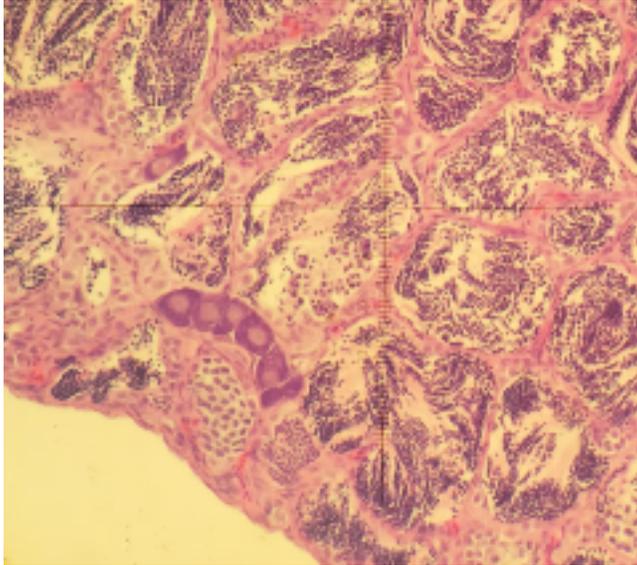
*Fish treated for 4 days and grown out in control water for 3 additional days before sacrifice

Appendix F: Histological Documentation of TO in LMB

Specimen: C1-21 Exposure: 1

Age at Exposure: 12 mph Age at Sacrifice: 21 mph

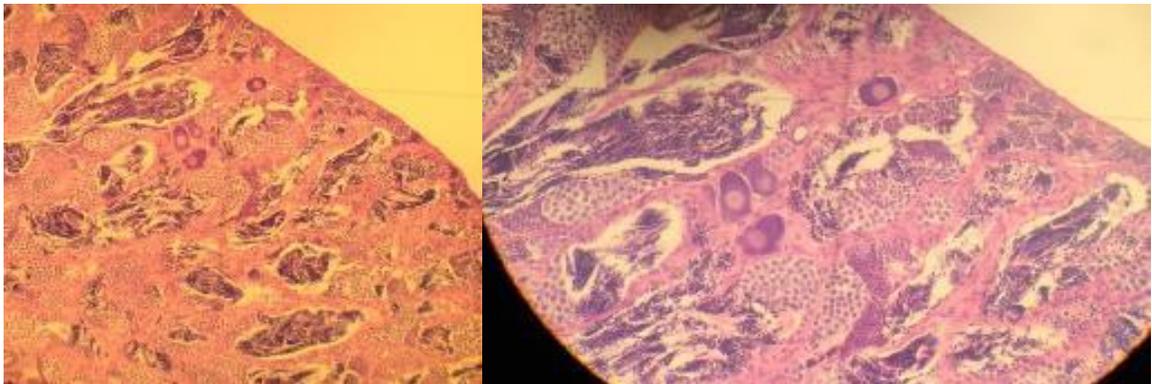
Body Section: Anterior Number of oocytes: 8



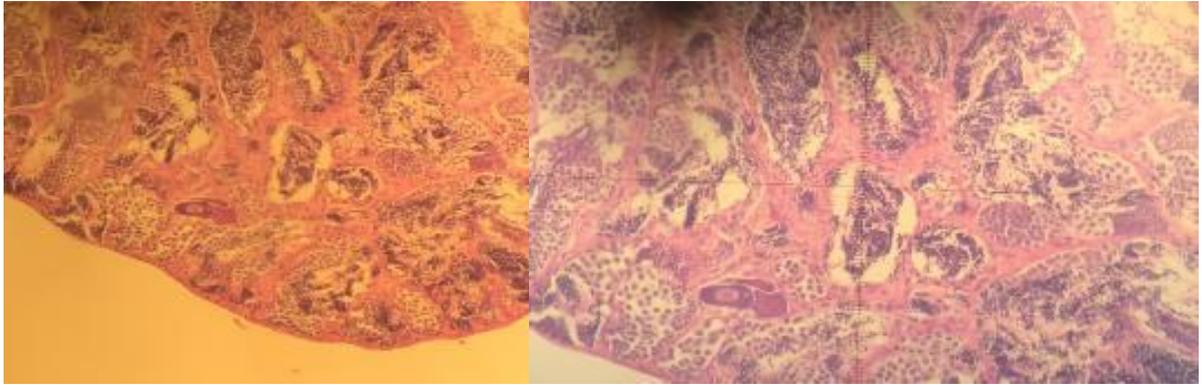
Specimen: C1-22 Exposure: 1

Age at Exposure: 12 mph Age at Sacrifice: 21 mph

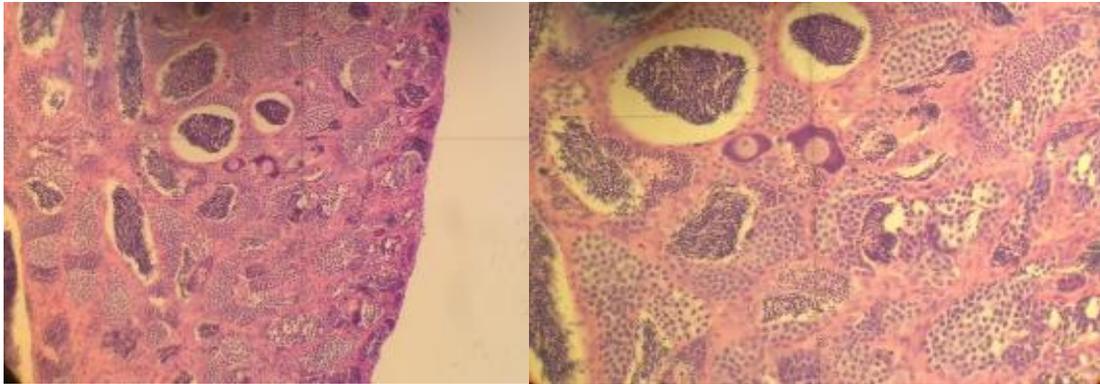
Body Section: Middle Number of oocytes: 4



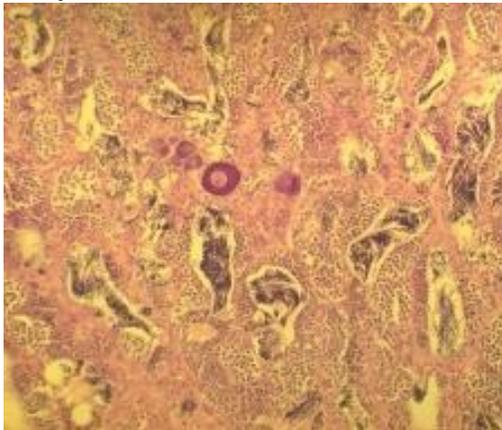
Specimen: C1-22 Exposure: 1
Age at Exposure: 12 mph Age at Sacrifice: 21 mph
Body Section: Posterior Number of oocytes: 3



Specimen: P1-44 Exposure: 1
Age at Exposure: 12 mph Age at Sacrifice: 21 mph
Body Section: Middle Number of oocytes: 2



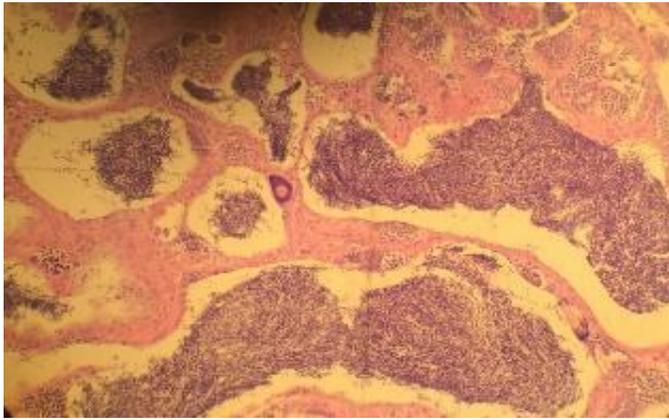
Specimen: L1-E3-35 Exposure: 3
Age at Exposure: 18 mph Age at Sacrifice: 21 mph
Body Section: Posterior Number of oocytes: 9



Specimen: L1-E3-41 Exposure: 3

Age at Exposure: 18 mph Age at Sacrifice: 21 mph

Body Section: Posterior Number of oocytes: 1



Trematode parasites found in 3 mph LMB from Exposure 2



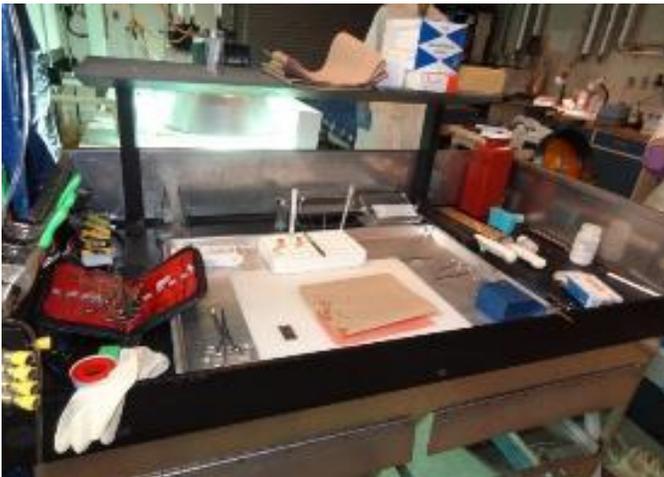
Appendix G: University of Maryland Animal Sciences Aquaculture Facility



Appendix H: Fish Plasma and Gonad Collection



Fish were first anesthetized with MS-222



A typical sacrifice station in Dr. Yonkos' Aquatic Toxicology Laboratory.



Blood was collected using capillary tubes after an incision was made in the caudal region.



Mature female gonad tissues were extracted and preserved whole for later histological preparation.



Mature male gonad tissues were extracted and preserved whole for later histological preparation.