ABSTRACT

Title of Dissertation: PHYSIOLOGICAL RESPONSES OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*) TO ANTHROPOGENIC SOURCES OF ENDOCRINE DISRUPTORS

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Anthropogenic sources of endocrine disrupting compounds (EDCs) are prevalent in aquatic ecosystems. As a result, these compounds target the most vulnerable physiological mechanisms of reproductive development in fish. The main focus of this study was to assess the impact of two sources of EDCs; a single potent EDC, 17α-ethinylestradiol (EE2), as well as an agricultural source of an EDC mixture, poultry litter leachate, on the reproductive processes of the fathead minnow, *Pimephales promelas*. The experimental paradigms included a 21-day laboratory reproductive assay, a larval exposure including a depuration step until 8 months of age to determine the response in the adult animal, and a whole-organism, partial life-cycle assessment of reproductively active adult fish and development of their offspring.

In the first experiment, breeding groups of fathead minnow were exposed to 0, 10, and 40 ng/L of EE2 for 21 days. A significant decrease in testosterone (T), the 17β-estradiol (E2)/T ratio and 11-ketotestosterone in males was observed in the 10 ng/L and 40 ng/L EE2 treatments compared to the control. Male secondary sex characteristics declined with increasing concentrations of EE2. Moreover, 43% of the males in the highest EE2 group (40 ng/L EE2) developed ovipositors. In experiment two, E2 was detected at approximately 200-400 pg/mL and T at approximately 100-400 pg/mL in the poultry litter leachate. Gender ratio was skewed toward female in the two highest concentrations of the poultry litter leachate treatment groups. In experiment three, with a transgenerational design, adult fish had reduced fecundity and males had a significant reduction in male traits when exposed to poultry litter leachate. Vitellogenin production was observed in male fish. In the offspring, both the Low and E2 fish from the same treated adults had significantly lower GSI values and aromatase activity levels. Exploration of different developmental stages and exposures across generations improves our understanding of the impact anthropogenic EDCs can have on wild fish populations.

PHYSIOLOGICAL RESPONSES OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*) TO ANTHROPOGENIC SOURCES OF ENDROCRINE DISRUPTORS

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

To my mother; for giving me your strength, your love of nature and living things, and the desire to always learn more.

To my father; for giving me your bravery, your passion, and your perseverance in all you do.

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Abstract

The pharmaceutical product 17α-ethinylestradiol (EE2) is a potent estrogen mimic present in the aquatic environment that poses significant risk to aquatic organisms, particularly fish. A reproductive assay was conducted using the fathead minnow exposed to environmentally relevant levels of EE2 to study effects on reproductive endpoints known to be hormone dependent. Condition factor was significantly reduced for fish exposed to the 40 ng/L EE2 treatment. Vitellogenin induction in males was also significantly expressed in both the 10 ng/L and 40 ng/L EE2 treatments. An observed decrease in plasma testosterone (T), the 17β-estradiol (E2)/T ratio and 11-ketotestosterone in males were significant in the 10 ng/L and 40 ng/L EE2 treatments compared to the control. Male secondary sexual characteristics such as the number of breeding tubercles and the epithelial pad thickness also declined with increasing concentrations of EE2. Moreover, 43 % of the males in the highest EE2 group (40 ng/L EE2) developed ovipositors, a female secondary sex characteristic. Fertility and fecundity were not significantly affected by EE2; however fecundity increased over the course of the 21-day study in the 10 ng/L EE2 treatments. Especially because observed adverse responses in males, the primary caregivers for fathead minnow, EE2 may adversely effect populations over time.

Keywords: 17α-Ethinylestradiol Fathead minnow Reproductive fitness

1. Introduction

Anthropogenic sources of toxic compounds released into the environment are a major threat to the aquatic environment. Many of these man-made compounds function as endocrine disrupting chemicals (EDCs) due to their ability to exert effects on the endocrine system in vertebrates. Examples of these EDCs include organochlorine pesticides, polychlorinated biphenyls, dioxins, and alkylphenols (van den Belt et al. 2002; Harries et al. 1996). Despite the fact that some of these compounds have not been utilized for years or even decades, these compounds are inherently insoluble in water, and have remained in the aquatic ecosystems, thus continuing to harm the physiology of aquatic organisms (Johnson and Williams 2004; Kolpin et al. 2002).

Many of these compounds exert their effects by mimicking the endogenous estrogens present in all vertebrate systems (Ankley and Johnson 2004). The primary role of estrogens is control of reproduction. However, when aquatic organisms are exposed to estrogen mimics in rivers, streams, and other lentic and lotic systems via sewage treatment facilities and non-point-source discharge sources (Harries et al. 1996), these compounds stimulate the reproductive system and may elicit a response which would normally be triggered by endogenous estrogens (Devlin and Nagahama 2002). There is considerable evidence that one of the leading contributors to feminization of some wild fish populations is 17α-ethinylestradiol or EE2 (Larsson et al. 1999; Desbrow et al. 1998; Jobling et al. 1998; Tyler and Routledge 1998; Harries et al. 1996).

1.1 Environmental consequences of EE2

EE2 is a synthetic estrogen found in many forms of the oral contraceptive pill. Its high potency, low solubility, and abundance in aquatic systems make it easily detected in

surface waters. EE2 has been found downstream from sewage treatment works because many of these facilities are not designed to remove EE2 from the effluent (Nash et al. 2004; Kolpin et al. 2002). The mechanism of action of this synthetic estrogen is purposefully to bind to the estrogen receptor and elicit a response on the reproductive system, similarly to endogenous estrogens. Oral contraceptives like the combination pills have EE2 and norgesterol or other hormones that act together to inhibit ovulation by suppressing release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), respectively (Frye 2006). As a result, pregnancy is blocked by sustaining the luteal phase of the menstrual cycle when the endometrium is thin and therefore prevents implantation of a fertilized egg (Frye 2006).

Due to the large percentage of western populations that ingest EE2 for contraceptive purposes, (17% of the total female population according to the U.S. Center for Disease Control 1995), there is the potential for a considerable amount of EE2 to enter the environment. As EE2 is metabolized and conjugated in the liver, approximately 27% or 7.1 μg/d of the 20-35 μg of EE2 in each dose is excreted into the urine, mostly in the glucuronide conjugate form (Johnson and Williams 2004). However, 30% of EE2 is excreted in the feces and most of this EE2 excreted (77%), is present in the non-conjugated, free, parent form. This then translates to 0.89 μg/d of EE2 being released into the environment per individual in the population (Johnson and Williams 2004).

The potency of EE2 is much greater than that of the primary endogenous estrogen, 17β-estradiol (E2). EE2 can bind to steroid hormone receptors, triggering subsequent responses on the reproductive system (van den Belt et al. 2002). Indeed, EE2 has a 10-50 fold higher binding affinity in fish than that of E2 and E1 (estriol) (Segner et

al. 2003). EE2 has a notably long half-life (~7 days in soils and in water) and a greater tendency to bioconcentrate in tissue than E2 or E1 (Länge et al. 2001). In Europe, EE2 is present in effluent and surface waters originating from sewage treatment works at concentrations between 0.5 and 7 ng/L but can reach levels as high as 50 ng/L (Larsson et al. 1999; Ternes et al. 1999; Desbrow et al. 1998). In North America, concentrations have been measured up to 273 ng/L, but average values were ~ 5ng/L at most sites tested (Kolpin et al. 2002).

Previous studies have investigated the effects of EE2 on the development of aquatic organisms and subsequent effects on reproduction, growth, immunity, and overall health as these organisms enter adulthood (Schäfers et al. 2007; Fenske et al. 2005; Nash et al. 2004; van Aerle et al. 2002; van den Belt et al. 2002; Länge et al. 2001; Zillioux et al. 2001; Scholz and Gutzeit 2000; Larsson et al. 1999; Purdom et al. 1994). The concern with EE2 exposure to aquatic organisms, particularly fish, is related to sexual development and differentiation (van Aerle et al. 2002). Previous research has shown that very low, environmentally relevant concentrations, 0.1-10ng/L of EE2, can impair gonadal cell differentiation, fecundity, fertilization, viability of embryos, skew gender ratios in populations, and alter behaviors associated with successful reproduction (Pawlowski et al. 2004; Hill and Janz et al. 2003; Segner et al. 2003; Majewski et al. 2002; Länge et al. 2001; Scholz and Gutzeit 2000). Thus, EE2 may have serious impacts on wild fish populations, potentially harming the success of future generations as well as the diversity of fish populations within an ecosystem (Kidd et al. 2007).

1.2 The hypothalamic-pituitary-gonadal axis in fish

Appropriate levels of circulating sex hormones in fish are dependent upon the proper signaling along the hypothalamic-pituitary-gonadal axis, a physiological system present in all vertebrates which drives the signals from the brain to other target systems to synthesize and release hormones necessary for reproduction (Devlin and Nagahama 2002). In fish, the initiation of reproductive events begins with an external (photoperiod, temperature, etc.) or internal stimuli (i.e., percentage of body fat) to send the appropriate signals to sensory organs such as the pineal gland (Sullivan et al. 1997). The function of the pineal gland is to secrete the neurotransmitter, melatonin in response to diel light cycle changes (Nelson 2000). Melatonin inhibits the secretion of the gonadotropic hormones from the pituitary gland via synthesis of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Khan and Thomas 1996; Nelson 2000).

As GnRH is released in fish, it triggers the synthesis and release of two major gonadotropins. One is similar to FSH that is found in other vertebrates which primarily induces oogenesis and spermatogenesis. The other gonadotropin is similar to luteinizing LH, whose primary activity causes final gamete maturation and induction of ovulation and sperm release (Ankley and Johnson 2004). The neurons extending from the hypothalamus into the pituitary control the release of FSH and LH through a negative feedback loop (Powell et al. 1996).

The pituitary gonadotropins bind to receptor sites located in cells at two different locations within target organs such as the gonadal tissue (Peter and Yu 1997). Binding at transmembrane receptor sites within testicular or ovarian tissue cells triggers the synthesis and release of steroid hormones such as estrogens and androgens (Peter and Yu

1997). Binding occurring within the nucleus of estrogen or androgen responsive cells will take place on hormone responsive elements on specific regions of the DNA. This triggers specific mRNAs to produce proteins that trigger steroid modulated growth such as development of secondary sex characteristics or gonadal cell proliferation (Beato et al. 1996).

The steroid hormone synthesis pathway begins with the metabolism of cholesterol via the cholesterol side-chain cleavage enzyme to be further metabolized to the C-19 compounds; the androgens. Further enzymatic breakdown of the C-19 androgens to the C-18 compounds, the estrogens, is done so via the aromatase enzyme in the gonadal tissue and the brain of teleost fishes (Tchoudakova and Callard 1998).

In the female fish, the specific localization of aromatization of androgens to estrogens in gonadal tissue takes place in the granulosa cells which surround the developing oocyte. Granulosa cells are also responsible for helping the oocyte grow and develop into a mature ovum (Peter and Yu 1997). The theca cells are a secondary cell layer surrounding the developing oocyte and these cells are responsible for synthesizing androgens which are then transferred to the granulosa cells to be aromatized (Peter and Yu 1997). The oocyte also contains receptors located on the outermost cell membrane where vitellogenin is absorbed into the cytoplasm during maturation in response to the production of estrogens (Peter and Yu 1997).

In the male fish, the testes consist of interstitial and tubular compartments where androgen production occurs in the Leydig cells. The main androgens produced,11-ketotestosterone and testosterone, are responsible for stimulating male characters, behaviors, and spawning events (Borg 1994; Nagahama 1994). The testicular cysts or

tubular component of the testes consist of two cell types, germ cells and Sertoli cells, where sperm develop and mature (Nagahama 1994). Steroid hormone synthesis and release from the respective cell types in the male and female are dictated by levels of hormones present circulating in the blood stream (Nelson 2000). A pulsatile pattern of release for these hormones allows the receptors to which the steroid hormones are binding to replenish once hormones have reached target organs. A physiological response can then be elicited during the period of feedback signaling (Nelson 2000). The presence of a sufficient level of circulating steroids in the blood is then interpreted as a signal to be sent back to the hypothalamus to control release of or suspend release of GnRH which in turn signals the pituitary gland for the controlled release of FSH and LH. This negative feedback mechanism is essential for the proper functioning and timed occurrence of events necessary for successful reproduction (Sullivan et al. 1997).

The initiation of sexual differentiation in fishes occurs just after hatching (Devlin and Nagahama 2002). Thus fishes are extraordinarily vulnerable to the effects of EDCs during this stage of development (Devlin and Nagahama 2002). The process of sexual differentiation relies upon, among other physiological processes, the activity of the aromatase enzyme to convert androgens to estrogens, thereby creating the proper ratio of each steroid hormone to discriminate gender (Simpson et al. 1994). In the adult animal, the activity of the aromatase enzyme is also critical to initiation of reproductive maturity and associated behaviors (Simpson et al. 1994). Thus, the activity of the aromatase enzyme may be affected by exogenous endocrine exposure during different stages in the reproductive development of an aquatic organism.

1.3 Fish response to EE2

Studies utilizing EE2 as an EDC source have focused on appropriate endpoints related to reproductive systems for a variety of fish species (Fenske et al. 2005; Nash et al. 2004; Pawlowski et al. 2004; van den Belt et al. 2002; Länge et al. 2001; Zillioux et al. 2001). For example, fertility, fecundity, and hatching of offspring have been used to assess the ability of these animals to reproduce successfully (Fenske et al. 2005; Nash et al. 2004; Pawlowski et al. 2004; van den Belt et al. 2002; Länge et al. 2001; Zillioux et al. 2001). In addition, some studies assessed reproductive hormone levels to determine if androgens and estrogens are at levels required for reproductive function. The fish species chosen were based on the ease with which they can be reared under laboratory conditions, whether they have a variety of secondary sex characteristics known to be under control of circulating hormones and whether they reproduce relatively rapidly in brief period of time (Nash et al. 2004; Ankley et al. 2001; Länge et al. 2001; Sohoni et al. 2001; Zillioux et al. 2001).

The fish species I chose to use for my study was the fathead minnow. The fathead minnow model was chosen because there is a well-established knowledge base for this species and the biology, ecology, and toxicity response to a large number of compounds, including EDCs, is well known. The fathead minnow is a fractional spawner and will produce numerous clutches of eggs every 3-5 days throughout the breeding cycle (Jensen et al. 2001; Gayle and Buynak 1982). For this reason, reproductive performance is easily assessed. In addition, the breeding strategy of these fish dictates that each male secure and defend a territory to guard as a potential egg-laying area (Cole and Smith 1987). Thus, the response of males to EDCs may impact reproductive performance.

Previous studies have shown that levels of EE2 between 0 and 10 ng/L caused reductions in fecundity, gonad weight, fertility, male reproductive behavior, spermatogenesis, and population numbers over time for fathead minnow and other species (Kidd et al. 2007; Nash et al. 2004; van Aerle et al. 2002; Scholz and Gutzeit 2000). In addition, the studies that used EE2 concentrations from 50-100 ng/L or greater showed reduced hatching, gill damage, testes-ova (evidence of sex-reversal), reductions in male secondary sex characteristics, and reduced condition factor for other species including the fathead minnow (Pawlowski et al. 2004; Länge et al. 2001; Zillioux et al. 2001; Scholz and Gutzeit 2000). My focus was to use environmentally relevant exposure concentrations but to maximize and integrate the physiological endpoints that were most relevant to successful reproduction in this species. I hypothesized that exposure to EE2 in the fathead minnow would disrupt the reproductive physiology and performance of the fathead minnow in terms of fertility, fecundity, and hatch success which in turn are incorporated with steroid hormone levels, aromatase activity, and condition factor. My specific objectives for this study were to 1) determine the impact of environmentally relevant levels of EE2 on the overall reproductive capacity of breeding groups of fathead minnow, 2) assess the extent of endocrine disruption on androgen-driven male traits with exposure to EE2, 3) quantify sexually dimorphic endpoints such as aromatase activity, steroid hormone levels and ratios of these steroid hormones with exposure to EE2, and 4) measure VTG induction as a biomarker for estrogenic exposure of male fish. This was a laboratory based exposure using mesocosms to assess the response of adult males and females to varying concentrations of EE2.

2. Materials and Methods

2.1. Animal care

Prior to initiation of the study, breeding groups consisting of one male and three females were placed into each tank with two pieces of 6.4 mm thick PVC pipe cut in half (see below). If egg production was sustained (spawning occurring every 3 to 4 days, a minimal fecundity of 15 eggs/female/day, >90% fertility; Ankley et al. 2001; Jensen et al. 2001) over the course of the two week acclimation period, the fish would remain for the experiment. If fish did not perform during the course of the acclimation period, they were replaced with new fish and a similar evaluation of reproductive potential would occur (Ankley et al. 2001). Adult male and female fathead minnow were randomly assigned to fifteen, 21 L tanks from the general population in the Aquatic Pathobiology Laboratory, College Park, MD. All animals were between 5-7 months of age and were chosen based on the presence of secondary sex characteristics for the males (distinct breeding tubercles, dorsal epithelial pad, and body condition). Females were chosen based on visual inspection of overall external health (robust body condition, active eating, no visual lesions or signs of stress). Each tank was provided with an airline using a plastic pipet to allow for gentle aeration of the water and sufficient oxygenation. Animals were maintained at 25±2° C and a photoperiod of 16:8 light:dark cycle.

Animals were fed twice daily either a slow-sinking pelleted #2 crumble diet (Finfish Starter #2, Ziegler Bros., Inc. Gardners, PA) or 1 mL of frozen brine shrimp slurry. Water changes (75%) were made each day therefore water quality was measured once per week in representative tanks from each treatment. Each water quality parameter

was measured prior to a water change. Temperature, pH, and concentrations of NH_3, NO_2, NO_3 were recorded.

2.2 Study design and treatments

The study was performed in the Spring of 2006. The experimental design was a CRD (completely randomized design; treatments assigned at random to experimental units) with three treatments consisting of 0 ng/L (0.001% ethanol), 10 ng/L and 40 ng/L EE2. Each treatment consisted of five replicates for a total of fifteen tanks. Twelve tanks contained breeding groups which consisted of one male, three females, and one breeding structure made of 6.4 mm thick PVC pipe cut in half that measured approximately 110mm X 93 mm X 48 mm. The remaining three tanks had eight fish, four females and four males with no breeding substrate. These tanks served as surrogate tanks to provide animal numbers for tissue samples for statistical purposes. The EE2 exposure study was conducted for 21 days (Ankley et al. 2001).

Water was delivered to each tank from two separate holding carboys designated as either EE2 treatment carboy or control carboy. Each carboy was equipped with an 800 W heater, an air stone, and a 1/2 hp, 650 W Tsurumi pump. EE2 (Sigma-Aldrich, St. Louis, MO) was weighed to 1 mg and dissolved in 100 mL ethanol and sonicated for 20 minutes. Twenty-five aliquots of the stock solution were prepared in advance and stored in microcentrifuge tubes, filled with 850 μL of the EE2 solution, and frozen at -20° C. On the first day of the experiment, the tanks were already filled to capacity with only carbon-filtered tap water, therefore the amount of EE2 added to each tank to establish the 40 ng/L treatments was 832 μL. Each day after the first day, 75% of the volume in each tank was removed, leaving some of the EE2 in the tank. The tanks were then filled back to

capacity with EE2 prepared water (mixed for 25-30 minutes) with a concentration of 40 ng/L.

Each subsequent day of the study, 640 μL of EE2 were added to the EE2 carboy containing 160 L of the carbon-filtered facility water. After the volume of each 40 ng/L EE2 treatment experimental unit was replenished, the carboy containing the 40 ng/L EE2 was then drained through a carbon filtration unit to a volume of 40 L. The carboy was then refilled to capacity with carbon filtered tap water (160 L) to create the 10 ng/L treatment. The tanks designated as 10 ng/L were then siphoned, cleaned, and refilled the same way as according to the 40 ng/L treatment preparation methods. Water for the control treatments was pumped from the control carboy after the tanks were siphoned to remove particulate.

2.3 Sample collection and measurement

All sampling procedures were performed under an approved Institutional Animal Care and Use protocol at the University of Maryland, College Park. At the end of the twenty-one day exposure, animals were euthanized in buffered MS-222 (100 mg/L with 200 mg $NaHCO_3$/L). Blood was collected from each fish by making a vertical incision at the vent up to the lateral line. The blood was drawn into heparinized hematocrit tubes and centrifuged at 10,000 g for 5 min. Hematocrit and blood volume were recorded. Each hematocrit tube was scored with a scoring tool at the buffy coat/plasma barrier, then cut at the scored section. Plasma was then drawn from the hematocrit tube using a syringe and stored in microcentrifuge tubes at -80°C until further analysis. A 1 μL aliquot of plasma from all male fish was placed in separate microcentrifuge tubes with sodium citrate buffer (0.1M, pH 7.6) in a 1:100 dilution for VTG analysis (Brodeur et al. 2006).

Weights and lengths of all fish were recorded to the nearest 0.001 g and to the nearest .01 mm for condition factor analysis. Condition factor was calculated as weight (g)/ length $(mm)^3$ X 100.

Aromatase activity was analyzed using a method modified from Thompson and Siiteri (1974) and reported per mg of protein. Whole brains were dissected and weighed to the nearest 0.001 g, placed into 100 µL of a potassium phosphate buffer (0.1 M, pH 7.4), homogenized for 20 s using a Fisher® Powergen 125 homogenizer and then placed at -80°C until further analysis for aromatase activity. A 3 µL aliquot of homogenate was also set aside and diluted 1:10 for protein analysis. The protein assay was performed in duplicate on all brain tissue with a BCA Protein Assay Reagent Kit (Pierce Chemicals, Dallas, TX). Samples were prepared in a 96-well microtiter plate and analyzed at 570 nm on a spectrophotometric plate reader. Aromatase activity was calculated and then mg of protein were determined to standardize per brain homogenate.

VTG in males was analyzed using an ELISA for fathead minnow purchased from Biosense Laboratories, Inc. (Bergen, Norway). The VTG assay was validated for the fathead minnow prior to analyses according to methods previously described (Nilsen et al. 2004). Samples were run in duplicate at three dilutions, 1:50,000, 1:500,000 and 1:5,000,000. Testosterone and estradiol were analyzed in both male and female fathead minnow using ELISA kits purchased from Cayman Chemicals, Inc. (Ann Arbor, MI). Male samples were run in duplicate at two dilutions, 1:5 and 1:50. Females were run in duplicate at 1:10 and 1:100. 11-ketotestosterone (11-KT) was also analyzed in male fathead minnow using an ELISA kit purchased from Cayman Chemicals (Ann Arbor, MI). Samples for 11-KT were run in duplicate at 1:2500, 1:5000, and 1:10,000. In

addition, the ratio between estradiol and testosterone was also assessed. All hormone analyses were validated prior to testing using non-exposed male and female fathead minnow. Plasma was diluted with kit buffer or reagent to a range of dilutions based on literature values for each hormone (Maritnović et al. 2007; Jensen et al. 2001). Values with dilutions that fell in the middle of the standard curve were chosen as the appropriate dilution factors for the final assay. For the final assay, if values fell below the VTG or hormone ELISA MDLs (method detection limits), proxy values of ½ the MDL of each individual kit were substituted prior to statistical analysis.

For male secondary sex characteristics, individual breeding tubercles were counted under a dissecting scope (Jensen et al. 2001). Qualifications on epithelial pad presence were ranked according to methods previous described by Smith and Murphy (1974) and ranked on a scale from 0-3. Ranks were assigned according to extent to which the pad was developed where 0=no pad (undeveloped), 1=minimal pad thickness 2=moderate pad thickness and 3=marked pad thickness. Ovipositor presence was also noted for both males and females during sampling. The ovipositor is a fleshy protrusion located at the vent of female fathead minnow. It is used during spawning to place eggs onto a substrate. Previous studies have used an index of presence of this trait as a sensitive early response to endocrine disruption in males (Parrot and Blunt 2005).

To verify gender, each fish was dissected just beyond the pectoral fin and at the caudal peduncle, and placed in 10% buffered formalin for histological analysis. Histological preparation of gonadal tissue was performed according to methods previously described (Ankley et al. 2001). Sections (5 μm thickness) were made from the narrow portion of the tissue. Four sections were made with 1000 μm between each

section. Each fish was prepared with a hematoxylin and eosin stain (American Histo Labs, Gaithersburg, MD). Gender was verified by microscopic inspection of each slide.

To determine reproductive fitness, breeding substrates were observed daily for the duration of the study for the presence of eggs. If eggs were present, breeding substrates were removed and the eggs were counted using the naked eye and a handheld counter. The substrate was then placed in a separate hatchery vessel with clean, carbon filtered tap water. A new, clean substrate was then placed into the tank from which it was removed. Each hatchery vessel contained an airstone to provide gentle aeration and circulation of water. The airstone was placed underneath the substrate to minimize fungal growth on the eggs and to maximize hatching. Daily recordings on fungal growth, eyed-up stage, number hatched, and date of hatch were performed. These data were collected to obtain information on fertility, fecundity, and hatch success.

2.4 Statistical Analyses

All analyses were performed on all groups, both surrogate and breeding groups, except testosterone data, 17β-estradiol data, and 11-ketotestosterone data. These were analyzed for breeding groups only to get best estimates of these hormones under reproductive cycle conditions. Data for condition factor, aromatase activity, all steroid hormones, reproductive capacity, and vitellogenin were analyzed using a one-way ANOVA followed by Tukey's test for means separation. If data did not meet the ANOVA assumptions (i.e., were non-normally distributed) data were transformed using the appropriate transformation method. Log transformations were made for aromatase, testosterone, tubercle count, and 11-ketotestosterone data. A square root transformation was done for VTG data. Data for secondary sex characteristics in males were ranked and

then analyzed using an appropriate test for a non-parametric one-way ANOVA (Kruskal-Wallis). All data analyses were performed using SAS® 9.1 (Cary, N.C.) and evaluated for significance at $p \leq 0.05$.

3. Results

During the course of the study, no mortality was observed for adult fish and all water quality parameters fell within acceptable levels (U.S. EPA 1987). Breeding groups were evaluated for fecundity and fertility two weeks prior to the start of the study to demonstrate that all groups were reproducing successfully. All breeding tanks had laid at least 2-3 clutches of eggs that were fertilized and hatched. Data for hatch success is not presented because the assessment of this endpoint was confounded by a prominent fungal problem regardless of treatment. The growth of fungus on eggs became unmanageable during the course of the 21-day exposure period.

Condition factor was different between genders ($p=0.01$), therefore the analysis was done separately for males and females. Condition factor significantly differed among the EE2 exposure treatments for males ($F=5.64$; $df=2, 13.1$; Figure 1.1). Male fish exposed to the 40 ng/L EE2 treatment had a significant reduction in condition factor compared to the male fish in the control treatment ($p=0.01$). No significant differences were observed for females ($F=2.87$; $df=2, 12$; $p=0.10$; data not shown).

Protein standardized aromatase activity differed significantly among treatments for female fish only ($p=0.002$; Figure 1.2). A significant difference was found between the control and the 40 ng/L treatment females, where the 40 ng/L treatment had a 13 % significantly higher aromatase activity than the control ($p=0.004$). The 10 ng/L treatment females also had a 12 % significantly higher aromatase activity than the control

(p=0.003). Aromatase activity among treatments between genders was not significant (F=0.39; df=2, 62; p=0.68).

VTG production was significantly induced in males with exposure to EE2 (F=207.7; df=2, 13; p=0.02; Figure1.3). A significant induction of VTG was observed in the 10 ng/L and 40 ng/L treatment groups. Both values were significantly higher than the control ($p < 0.0001$ for both treatments). Male fish exposed to the 10 ng/L EE2 treatment had 33% greater levels of VTG than male fish exposed to the 40 ng/L treatment The sensitivity or LOQ of the assay was 2.2 mg/mL, and the MDL or detection limit of the assay was 0.09 mg/mL. Repeatability precision was 2%

The level of 11-KT was only measured in males. Specifically, a significantly reduced level of 11-KT was observed between the control and the 40 ng/L EE2 treatment (F=4.71; df=2, 9; p=0.04; Figure 1.4). E2 was measured in male fathead minnow only (Figure 1.5). No differences were found among treatments for breeding groups (p=0.99; F=0.40; df=2, 19). However, means for both the 10 ng/L and 40 ng/L EE2 treatments were 67% and 75% higher, respectively, than those values for the control group.

Testosterone (T) was measured in both male and female fathead minnows. Significant differences were found among treatments for male fish where the 10 ng/L and 40 ng/L were both significantly lower than the control male fish T levels (p=0.003, and p=0.005; Figure 1.6). Female fish in the 40 ng/L EE2 treatment had a significantly lower level of testosterone than control male fish but there were no differences with respect to T levels among female fish (F=1.20; df=2, 55; p=0.05 and p=0.06). The E2/T ratio was analyzed for males (F=1.32; df=2, 31; Figure 1.7). This ratio was significantly greater for

the 40 ng/L EE2 treatment (p=0.005) and the 10 ng/L EE2 treatment (p=0.005) than the ratio for the control.

The average number of breeding tubercles significantly differed among treatments for male fish (F=11.7; df=2, 18; p=0.0005; Figure 1.8). The 40 ng/L EE2 treatment had a significantly lower average number of breeding tubercles compared to the control (p=0.0004). The 10 ng/L EE2 group also had a significantly lower number of tubercles per male than the control (p=0.05). Wilcoxon scores for dorsal epithelial pad ranked thickness for treatments were as follows: 0 ng/L=11.85, 10 ng/L=9.13, and 0 ng/L=7.00 (Figure 1.9). No significant differences were found with respect to epithelial pad thickness (F=1.84; df=2, 16; Pr > Chi-Square=0.15). However, a trend in the data shows a decline in pad ranked thickness as EE2 concentration increases.

The development of female secondary sexual characteristics in males has been associated with exposure to estrogenic compounds in some studies (Seki et al. 2005; Melo and Ramsdell et al. 2001; Scholz and Gutzeit 2000; Nimrod and Benson 1998). Interestingly, in my study, several males were observed to have developed ovipositors (Figure 1.10). Although ovipositors were not present in males in the 0 ng/L or 10 ng/L treatment groups, ovipositors were present in 43% of males in the 40 ng/L treatment group.

Exposure to EE2 increased mean egg production (fecundity) at the 10 ng/L treatment level but suppressed mean egg production at the 40 ng/L treatment level, although these differences were not significant (p=0.47; Figure 1.11). However, fecundity and fertility were reduced in the 40 ng/L EE2 treatment where number of clutches produced (41% fewer) and 29 % fewer fertilized eggs were observed compared to

controls. Fertilization rates per clutch did not differ significantly across treatments where control fertility was less than the 10 ng/L EE2 treated groups but greater than the 40 ng/L EE2 treated groups ($p=0.70$; data not shown).

4. Discussion

Environmental pollution with natural or synthetic estrogen mimics may pose serious threats to reproductive fitness in wildlife species (van den Belt et al. 2002). A survey of surface waters conducted in the United States revealed 15.7 % of the 139 stream networks across 30 states contained > 5 ng/L EE2 (Kolpin et al. 2002). Other research has shown levels as high as 42 ng/L EE2 detected in North American waters (Ternes et al. 1999). Previous studies have established that various reproductive parameters of fish, when utilizing similar or lower concentrations of EE2, were adversely affected (Fenske et al. 2005; Nash et al. 2004; Pawlowski et al. 2004; Seki et al. 2003; van den Belt et al. 2002; Länge et al. 2001; Zillioux et al. 2001).

I evaluated the effects of EE2 using several reproductive endpoints of the fathead minnow in laboratory based experiments. The levels of EE2 used (10 and 40 ng/L) were both environmentally relevant and biologically active levels of EE2, with the goal of demonstrating the potential impact of this compound in the aquatic environment (Kolpin et al. 2002; Ternes et al. 1999). The most significant results I observed from my study were those among male fathead minnow. Males produced a significant amount of VTG, as well as a significant reduction in critical male hormones 11-KT and T. In addition, males in the 40 ng/L EE2 treatment lost a significant amount of breeding tubercles and developed ovipositors, a female characteristic.

Biomarker tools are essential in assessment of potential impacts observed from a variety of stressors (Zhang et al. 2007; Brion et al. 2004; Ankley et al. 2003; Schmitt et al. 1999; Folmar et al. 1996). The use of VTG expression as a robust and direct measure of estrogenic exposure has been used extensively to note changes in the physiology of an organism (Fenske et al. 2005; Seki et al. 2005; Nash et al. 2004; Seki et al. 2003; van den Belt et al. 2002; Sohoni et al. 2001; Tyler et al. 1999; Harries et al. 1997; Harries et al. 1996; Sumpter 1995; Purdom et al. 1994). VTG is also a very useful method in that induction can remain elevated for several days and is detectable even after the exposure source is removed (Korte et al. 2000). VTG is a phospholipoglycoprotein produced in the liver of all oviparous vertebrates (Brooks et al. 1997). It is directly under the control of circulating estrogens and gets deposited into the developing oocyte as a nutritional source for the developing embryo. As such, it is normally only induced and measurable in females. However, males do carry the gene and it remains latent until triggered when exposure to an estrogenic substance occurs (Sumpter and Jobling 1995).

In my study, a significant induction of VTG in males was expressed with exposure to EE2. Despite the significant induction of VTG, it does not always appear to be predictive of impaired reproductive traits (Kramer et al. 1998). Exposure to EE2 and E2 has been shown to impair male gonadal tissue development and impair fertilization success at levels that may induce VTG but does not demonstrate any adverse effects on male secondary sex characteristics (Seki et al. 2005; Nash et al. 2004; Pawlowski et al. 2004; Miles-Richardson et al. 1999).

There is an extensive knowledge base regarding the reproductive physiology of the fathead minnow for hormone analyses. Levels of 11-ketotestosterone in males varies

from 30-40 pg/mL (Martinović et al. 2007; Ankley et al. 2003) and in females has been measured as non-detectable to 360 pg/mL (Ankley et al. 2003; Jensen et al. 2001). Androgens in fishes are responsible for stimulating male characters, behaviors, and spawning events (Borg 1994; Nagahama 1994; Demski and Hornby 1982). The hormone 11-KT is the predominant androgen in teleost fish and various behaviors of male fish including territoriality, nest-building, and courtship are strongly influenced by 11-KT (Mayer et al. 2004; Loomis and Thomas 2000; Borg 1994).

I found levels of 11-KT were significantly reduced with exposure to 40 ng/L EE2. Because male and female fish rely heavily on obligatory levels of 11-KT during the breeding cycle, a significant reduction of 11-KT observed with EE2 exposure could have serious impacts on the ability of males to perform reproductive behaviors and females to respond accordingly. A compromised breeding display could in turn result in less egg production or rather, less protection for a clutch of eggs over time.

Circulating levels of estradiol and testosterone in actively spawning male fathead minnow have been found to be non-detectable (Ankley et al. 2003) and in a range of 9-12 ng/mL (Jensen et al. 2001). Levels for E2 and T in actively spawning female fathead minnow are in the range of 4-6 ng/mL (Ankley et al. 2003) and 3-5 ng/mL (Jensen et al. 2001). Although levels of E2 did not significantly differ among the EE2 treatments in males, I observed a trend towards increasing levels of E2 corresponded with increasing levels of EE2 exposure. Conversely, I observed T levels in males decreased significantly with respect to increasing levels of EE2 exposure. T levels in male and female fish were also reduced with respect to exposure to diethylstilbestrol (DES) and EE2 respectively, in the Chinese rare minnow (*Gobiocyprus rarus;* Zhong et al. 2005) and the zebrafish

(*Danio rerio*; Nash et al. 2004). With respect to levels of T in the controls in my study, endogenous T was expressed in a sexually dimorphic manner in which males expressed significantly more T than females (Ankley et al. 2003; Jensen et al. 2001).

Several other studies have utilized the ratio between circulating estradiol and testosterone as an appropriate measure of endocrine disruption (Orlando et al. 2004; Harries et al. 2000; Folmar et al. 1996; Goodbred et al. 1996). Because the ratio of estradiol to testosterone is critical in the developing animal as well as during reproductive maturation in the adult, this ratio has provided evidence for consideration as a reliable measure for EDC studies.

A significant increase in the E2/T ratio among male fathead minnow was observed in my study. Orlando et al. (2004) also found the ratio between E2 and T was significantly increased in male fish collected from a cattle feedlot pond and a stream receiving run-off from a cattle operation. Yet, care must be taken when comparing results from Orlando et al. (2004) because the source of the T and E2 was cultured from gonadal tissue, (E2 and T in my study were from plasma) and exposure to a potential EDC mixture like effluent with unknown estrogenic content may yield differential results than a singular exposure to the one very potent estrogen I utilized in my study. Nichols et al. (1999) also demonstrated a significant difference with respect to E2/T ratio in fathead minnow exposed to sewage treatment effluent. They observed that changes did not occur among females at any of the sites, but T levels in males and subsequently E2/T levels among males had occurred. This trend may implicate a potential estrogenic influence from the sewage treatment sites.

Because male characteristics are reliant upon circulating levels of steroid hormones that are susceptible to exogenous EDC exposure, reduced expression of these traits can indicate compromised reproductive potential (Majewski et al. 2002; Länge et al. 2001). Reproductively mature male fathead minnow develop breeding tubercles present in a bilaterally-symmetrical pattern around the eyes and between the nares (Jensen et al. 2001; Image 1.1). Tubercles are used as a defense mechanism to confront potential nest site commandeers (Ankley et al. 2001; Jensen et al. 2001; Unger 1983; McMillan and Smith 1974; Richardson 1937). Previous research involving EE2 exposure in other teleosts, such as the Japanese medaka (*Oryzias latipes*) and the zebrafish (*Danio rerio*), that express male secondary sex characteristics, has demonstrated an increase in EE2 exposure causes the expression of secondary sex characteristics in males to decline (Nash et al. 2004; Pawlowski et al. 2004; Hemming et al. 2001; Länge et al. 2001; Scholz and Gutzeit 2000). I observed a significant dose-dependent decline in tubercle numbers similar to what was observed by Nash et al. (2004) and Pawlowski et al. (2004) with EE2 exposure. Hemming et al. (2001) also demonstrated that fathead minnow exposed to domestic sewage effluent containing EE2, nonylphenols, and phthalates also experienced a significant decline in breeding tubercle number. Fewer breeding tubercles may indicate a decline in fitness of a reproductively mature male or indicate lack of breeding status overall. Subsequently, this may significantly reduce the ability of a male to contribute genetic information into the next generation (Martinović et al. 2007; Bjerselius et al. 2001; Bayley et al. 1999).

Levels of both androgens and estrogens are essential for reproduction and are controlled by the extent of estrogen synthesis from androgens by the activity of the

aromatase enzyme (Simpson et al. 1994). I found that aromatase activity was significantly elevated across treatments compared to controls in female fish only, however no differences were observed with respect to treatment effects among males or gender differences due to the large variation among groups. In a similar way, previous studies using E2 as the estrogen demonstrated increased gene expression of aromatase or aromatase activity in male medaka (*Oryzias latipes*) (Melo and Ramsdell 2001; Scholz and Gutzeit 2000). With exogenous exposure to an estrogenic substance, the response of the hypothalamic-pituitary-gonadal axis is to down-regulate release of subsequent hormones (GnRH or FSH/LH) depending on the stage of development, gender, and timing in the reproductive cycle. Observed elevations in aromatase activity may be related to the level of EE2; the response elicited by a higher amount of estrogen would be interpreted to up-regulate aromatase and subsequently convert more androgen. However, the lack of observed difference between genders may be an indication that both genders are experiencing such high levels of estrogen being metabolized that both males and females resort to expressing abnormally high levels of aromatase, regardless of gender.

Condition factor has been used extensively in fish health population assessments (Mukhi et al. 2005; Pyle et al. 2005; Pawlowski et al. 2004; Hemming et al. 2001; McMaster et al. 1991). This variable is a robust means to assess fish health in that it integrates many factors of sub-organismal processes and may signify the overall health and nutritional status of a given animal (Dethloff and Schmitt 2000). Other studies have shown condition factor can either increase (Adams et al. 1992; McMaster et al. 1991), decrease (Munkittrick and Dixon 1988), or not change (Gravel et al. 2005; Mukhi et al. 2005) with exposure to potential endocrine disrupting compounds. I found a decline in

condition factor only in male fathead minnow with exposure to the 40 ng/L EE2 treatment. Pawlowski et al. (2004) and Hemming et al. (2001) observed a similar decline in condition factor in fathead minnow exposed to concentrations of EE2 between 10 and 100 ng/L and with exposure to wastewater effluent containing EE2, nonylphenols, phthalates, and DDT. As condition factor indicates a general health or energy transfer assessment, a lowered condition factor may be indicative of organisms utilizing energetic processes not focusing on reproductive efforts and instead geared toward metabolic processes that have clearly been disrupted.

My results demonstrate that exposure of fathead minnow to EE2 at environmentally relevant concentrations is both biologically active and adversely impacts several physiological parameters which are essential for successful reproduction. Declines in condition factor, male secondary sexual characteristics, and particularly circulating levels of proper hormone ratios are cause for concern. For example, Kidd et al. (2007) demonstrated a population crash occurred after only two generations of EE2 exposure in the fathead minnow in a 7-year whole-lake dosing study using levels less than what I used in my experiment. Surprisingly, observed female secondary characteristics (ovipositors) in male fathead minnow exposed to 40 ng/L EE2 did not overtly alter reproductive performance in these males. However, the presence of this trait in males is cause for alarm and may be an additional useful biomarker.

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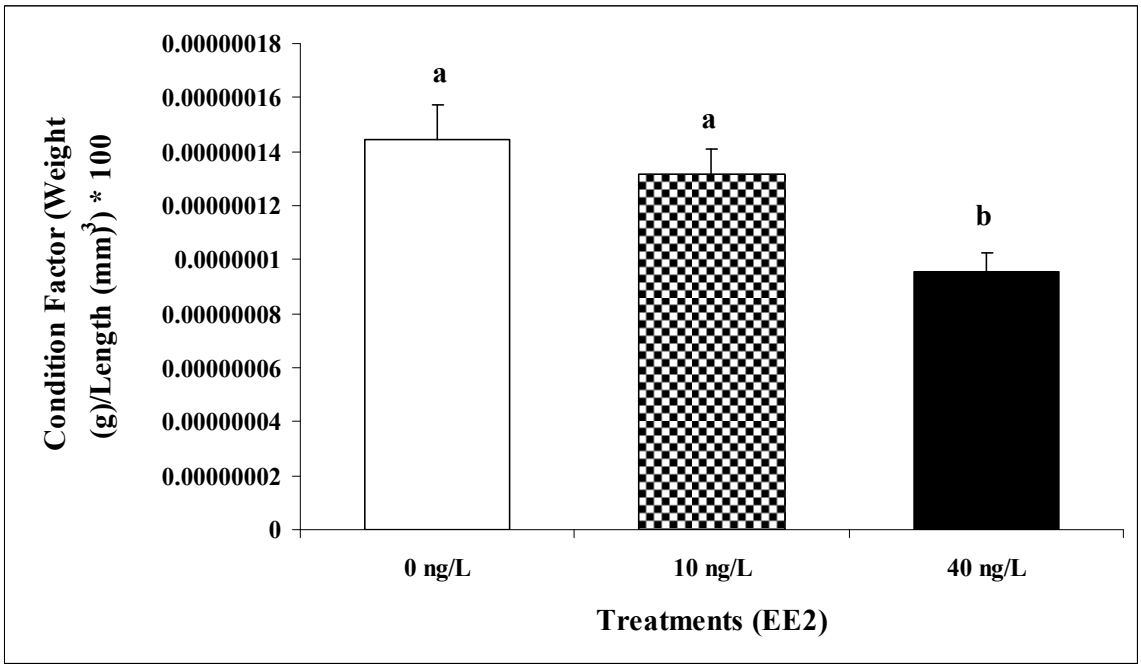

Figure 1.1. Condition factor among treatments for male fathead minnow exposed to EE2 for 21 days. Significant differences are indicated by different letters (p=0.01). Values are estimates and 95% upper confidence intervals (N=11-14 fish/treatment).

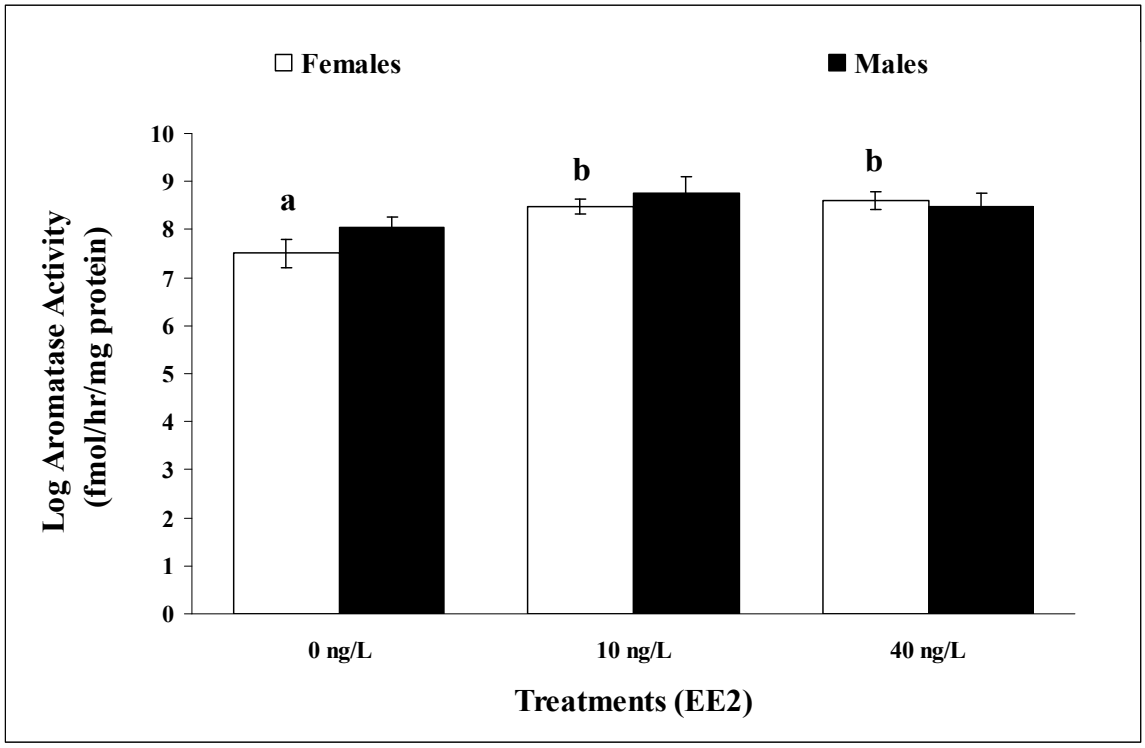

Figure1.2. Aromatase activity among treatments for fathead minnow exposed to EE2 for 21 days. Significant differences between means were only found among female fathead minnow control and EE2 treatments (p=0.01) and are indicated by different letters. Values are log transformed means ± SEM (N=21-24 fish/treatment).

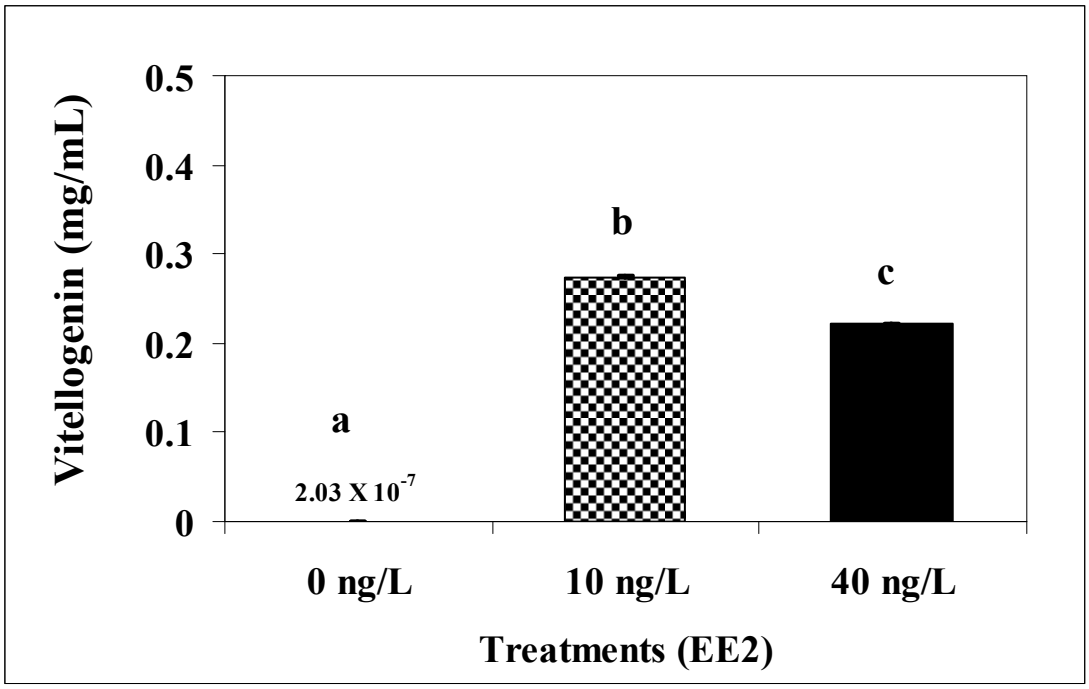

Figure 1.3. Vitellogenin induction in males exposed to EE2 for 21 days. Differences were found between the control and EE2 treatments ($p < 0.0001$) and between the 10 ng/L and 40 ng/L treatments ($p=0.02$). Differences between means are indicated by different letters. Values are means ± SEM (N=3-9 males/treatment).

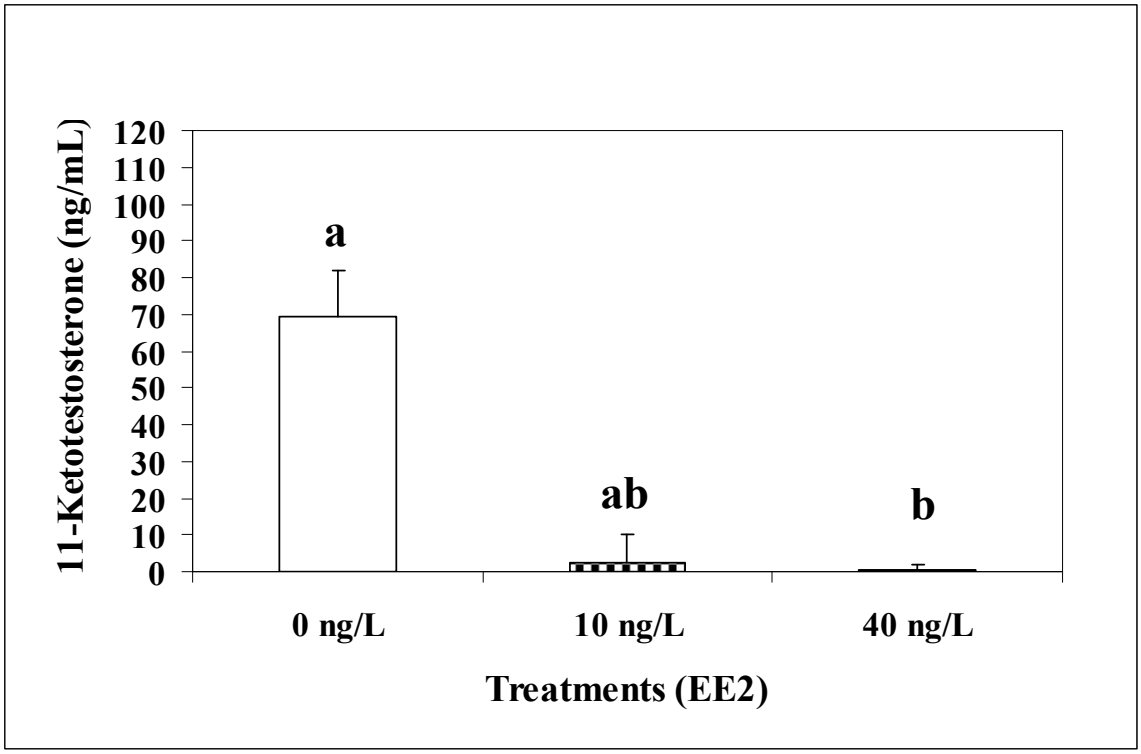

Figure 1.4. 11-ketotestosterone in male fathead minnow from breeding groups exposed to EE2 for 21 days. Differences were found between the control and the 40 EE2 treatment and are indicated by different letters (p=0.04). Values are estimates and 95% upper confidence intervals (N=4 males/treatment).

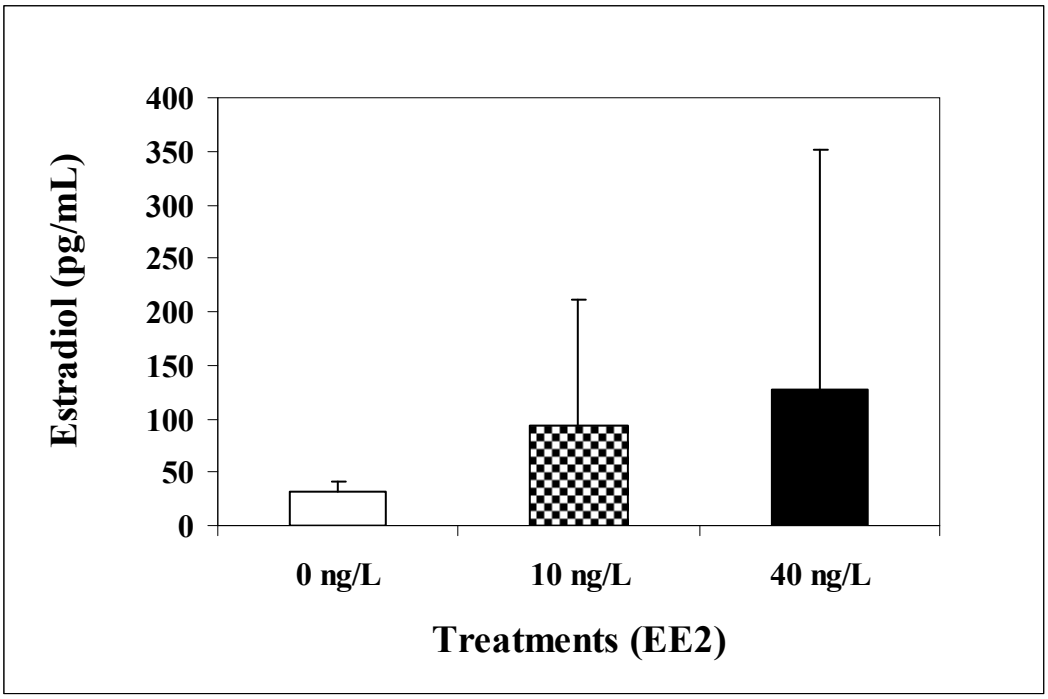

Figure 1.5. Estradiol levels in male fathead minnow in breeding groups exposed to EE2 for 21 days. Values are estimates and 95% upper confidence intervals (N=4-12 males/treatment; p=0.99).

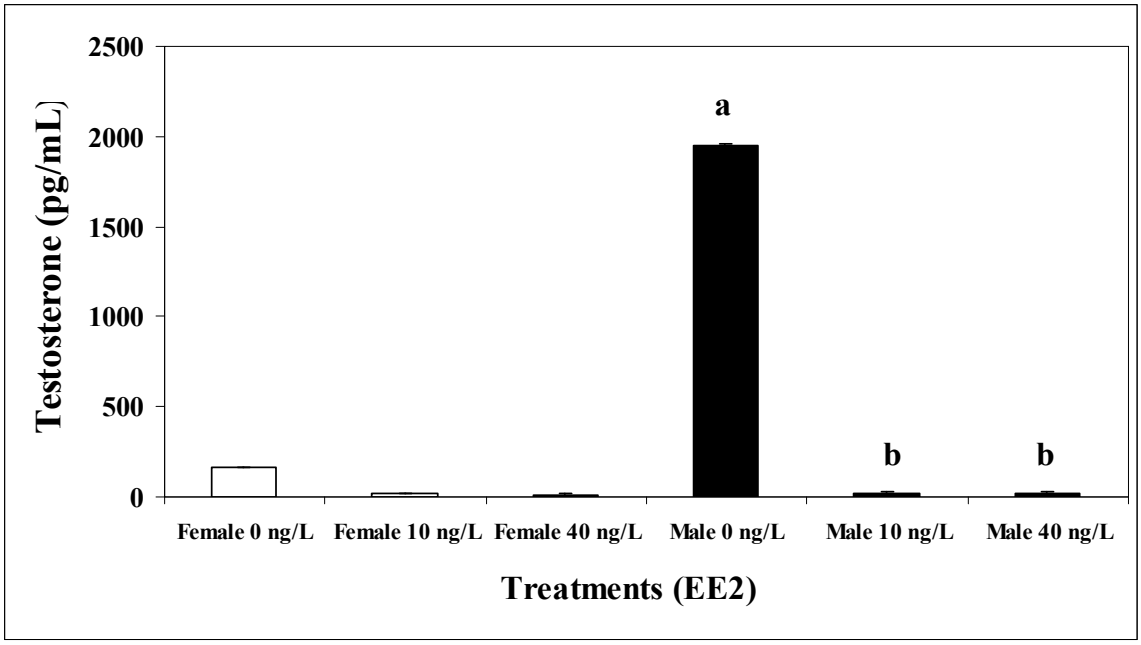

Figure 1.6. Testosterone (T) levels in male and female fathead minnow in breeding groups exposed to EE2 for 21 days. Differences were observed among genders (p=0.05), and differences among males are indicated with different letters. Control male T values were significantly different from both the 10 ng/L and 40 ng/L EE2 treatments (p=0.003 and p=0.005, respectively). Values are estimates and 95% upper confidence intervals (N=8-11 females/treatment, N=4-12 males/treatment).

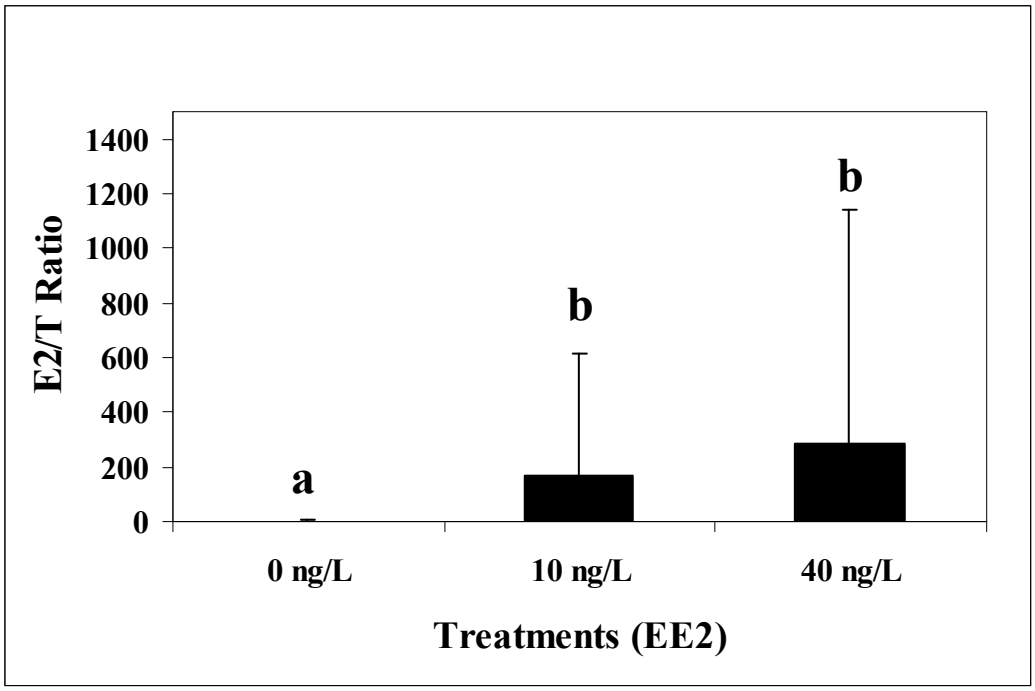

Figure 1.7. The ratio of E2 to T in male fathead minnow exposed to EE2 for 21 days. Values are estimates and 95% upper confidence intervals (N=4-12 males/treatment). Differences are indicated with different letters (p=0.005).

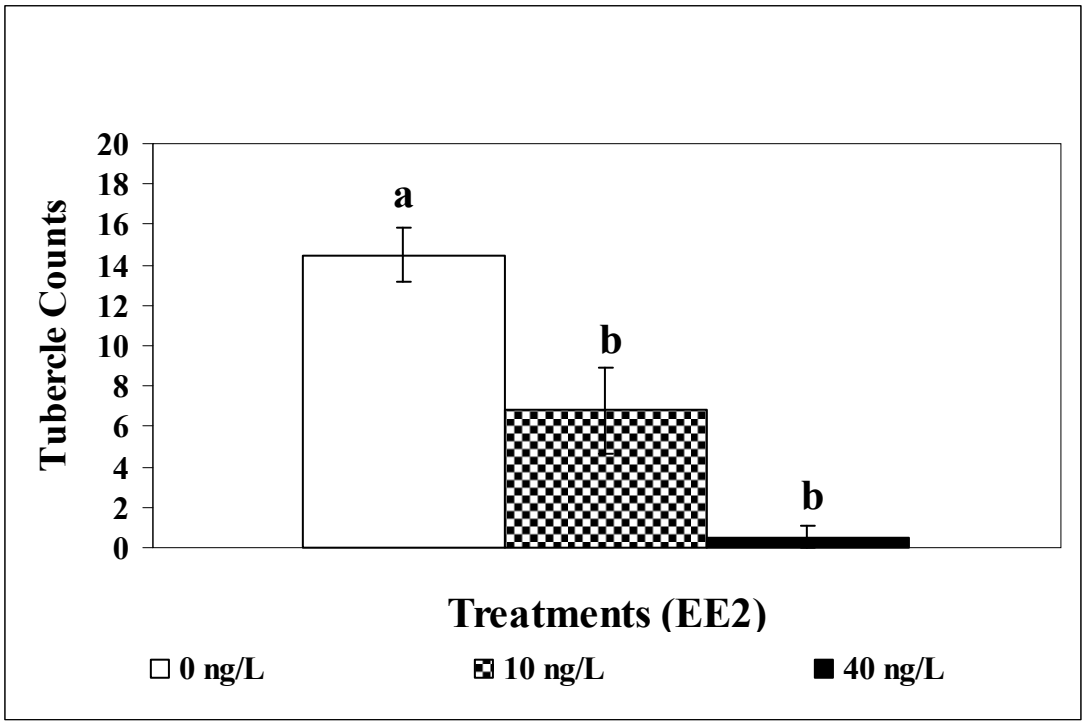

Figure 1.8. Average breeding tubercles per male in fathead minnow exposed to EE2 for 21 days. Differences are indicated by different letters. Control males had more breeding tubercles than the 10 ng/L EE2 treatment (p = 0.05) and the 40 ng/L EE2 males (p=0.0004). Values are means ± SEM (N=4-12 males/treatment).

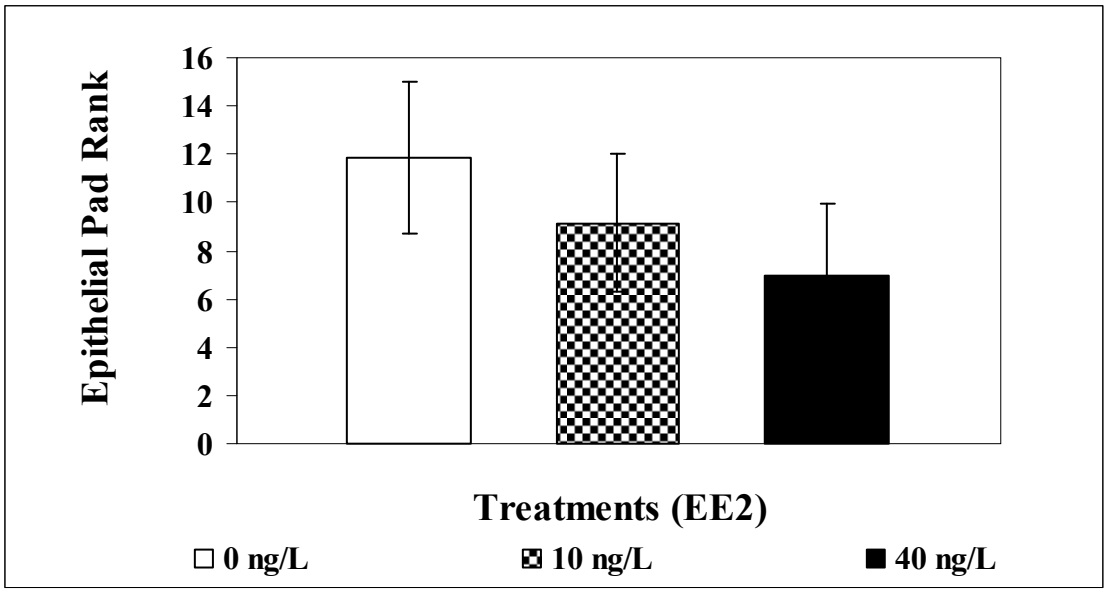

Figure 1.9. Epithelial pad rank in male fathead minnow exposed to EE2 for 21 days. Values are Wilcoxon scores (rank sums). N=4-12 males/treatment; differences between control males and 10 ng/L EE2 and 40 ng/L EE2 were not significant (p=0.20 and p=0.50, respectively).

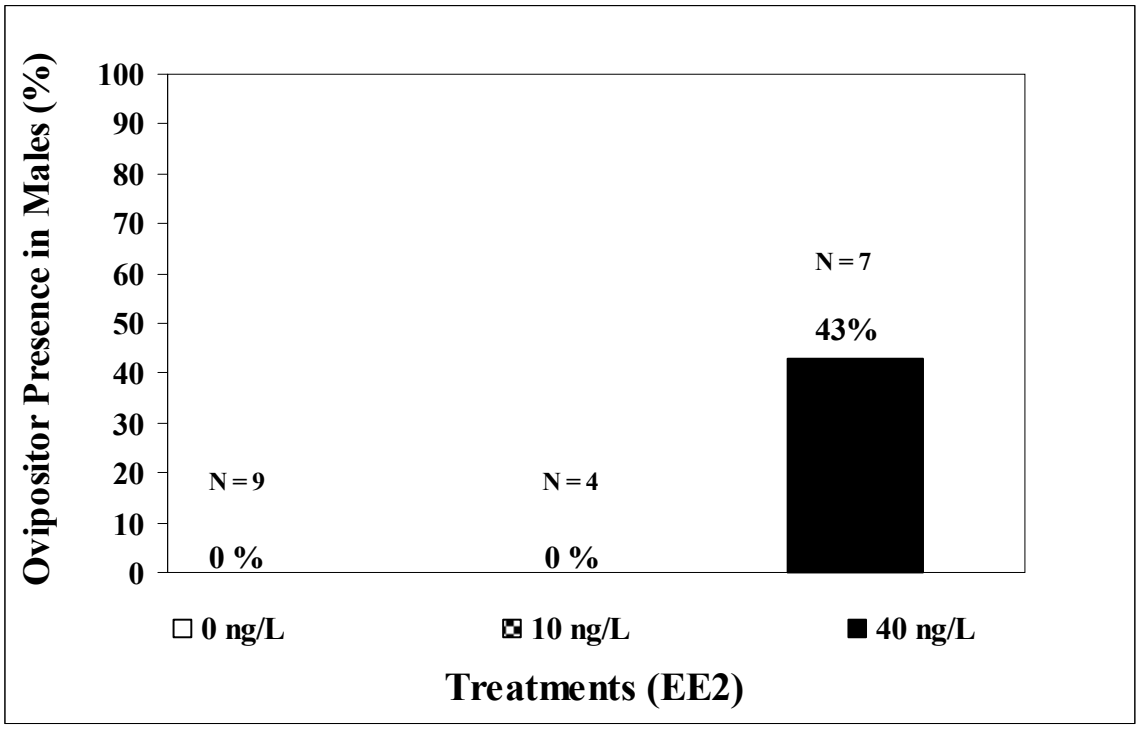

Figure 1.10. Percent of males exposed to EE2 for 21 days with ovipositors, a female secondary sex characteristic (N=4-9 males/treatment).

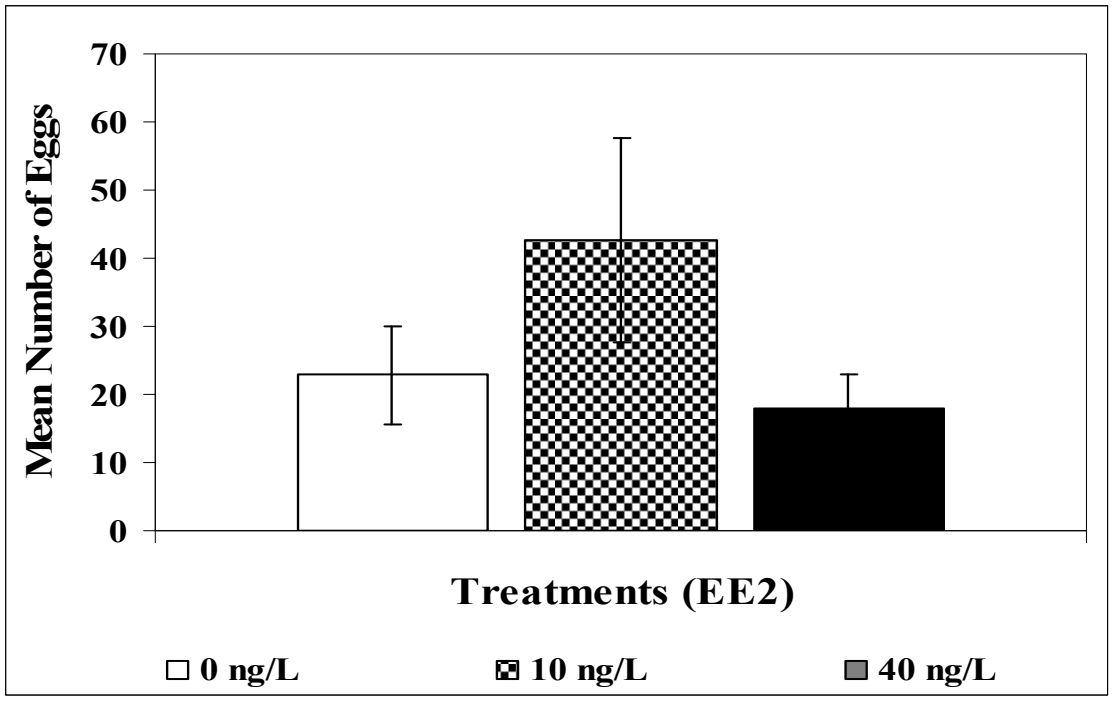

Figure 1.11. Mean egg production (fecundity) for EE2 exposed breeding groups of fathead minnow.

Image 1.1. Fathead minnow male breeding tubercles. Breeding tubercles are arranged in a radial symmetrical pattern on the face and are only present in reproductively mature males.

Chapter 2: Adult Response to Early Exposure of Fathead Minnow (*Pimephales promelas)* Larvae to Poultry Litter Leachate

Abstract

A significant source of endocrine disrupting contaminants (EDCs) in the Chesapeake Bay region may come from agricultural run-off from fields treated with poultry litter. The health of aquatic species could be affected, particularly during early development. In this study, larval fathead minnow was exposed to poultry litter leachate (PLL) at three concentrations, and biological endpoints were assessed when these animals reached maturity (eight months). Condition factor, aromatase activity, gender ratio and gonadal cell stage were recorded for both sexes. The presence of the dorsal epithelial pad and number of breeding tubercles were recorded for males. The PLL was analyzed prior to exposures to test for detectable levels of steroid hormones and to compare various preparation methods on PLL to determine how to maximize detectable hormones. Estradiol and testosterone were detected in the leachate at levels both environmentally relevant and biologically active (approximately 200-400 pg/mL estradiol and approximately 100-400 pg/mL testosterone). Analyses of secondary sex characteristics in males revealed a significant difference in expression of breeding tubercles in the intermediate level of the PLL; the 1:8 concentration. Gender ratio was skewed toward females in the two highest concentrations of PLL treatment groups; the 1:4 PLL concentration (88%) , and the 1:8 concentration (76%). No significant effects were observed in aromatase activity among treatments, however, differences were found between genders suggesting early exposure to PLL has no effect on the sexually dimorphic nature of the activity of this enzyme. This study is the first to investigate the

potential for exposure of larval fish to a complex EDC mixture such as poultry litter leachate, and to note the developmental response in the adult fish.

Key words: Larval exposure Poultry litter Endocrine disruption Fathead minnow

1. Introduction

The adverse effects of endocrine disrupting compounds (EDCs) on the reproductive axis in aquatic organisms is well documented (Tilton et al. 2005; Hornung et al. 2004; Nash et al. 2004; Ankley et al. 2003; Parks et al. 2001; Harries et al. 1997; Harries et al. 1996; Sumpter 1995; Purdom et al. 1994). EDCs are a collective of thousands of substances that interfere with the synthesis, secretion, action, and elimination of endogenous hormones when encountered in the environment (U.S. EPA 1998; Kavlock et al. 1996). Although the target systems for the effects of EDCs are well known, the mechanisms of action of many EDCs are poorly understood. Therefore, the activity of the endocrine systems as targets of these contaminants requires sensitive means to assess effects from exposure. This is particularly important as we become aware of more substances that can be considered EDCs. In addition, the ability of EDCs to exert effects at very low concentrations during critical, labile stages of development is also a concern (Bigsby et al. 1999; vom Saal et al. 1997; Colburn et al. 1993).

1.1 Early development in fishes

Evidence from wild populations of fish, amphibians and reptiles have shown EDCs detected at ng/L concentrations are associated with abnormal sexual differentiation and altered development in early life stages (Fenske et al. 2005; Seki et al. 2005; Hayes et al. 2002; Pickford et al. 2000; Nimrod and Benson 1998). Effects of EDCs have been studied on the life-cycle or partial life-cycle of a number of aquatic species (Bejerano et al. 2006; Seki et al. 2005; Zhong et al. 2005; Nash et al. 2004; Hutchinson and Pickford 2002). These exposure regimes assess effects throughout the life-cycle of the animal with continuous exposure.

In contrast, few studies have examined the effects of early exposure to EDCs on larval fish and then observe physiological impacts once these fish become adult (Fenske et al. 2005; McAllister and Kime 2003; Sohoni et al. 2001; Nimrod and Benson 1998). For many teleost species, the critical window for sexual differentiation is shortly after hatching (0-30 days post-hatch; dph), thereby rendering animals at this stage of development most vulnerable to the effects of EDCs (Koger et al. 2000; Scholz and Gutzeit 2000). Estradiol exposure during the critical period of development or *in ovo,* has been observed to skew the gender ratio of fish toward all female (Nash et al. 2004; Hill and Janz 2003; Scholz and Gutzeit 2000; Tyler e al. 1999). Therefore, addressing questions regarding EDC exposure in young, developing fish is critical to understanding the changes that can occur with EDC exposure during physiological development.

During early development, fish become sexually differentiated due to endogenously produced steroid hormones (Devlin and Nagahama 2002). Sexually dimorphic levels of circulating estrogens and androgens dictated by the activity of the cytochrome P450 aromatase enzyme (CYP19) is essential. In adult animals, the activity of this enzyme is equally critical to reproductive performance during the breeding cycle. If the function of the aromatase enzyme is inhibited, female fecundity declines dramatically (Fenske and Segner 2004). Subsequently, increased concentrations of plasma androgens are associated with aromatase inhibition and are associated with an all male population (Fenske and Segner 2004; González and Piferrer 2003; Ankley et al. 2002).

1.2 Endocrine disruptors in agricultural systems

The inherent plasticity of the fish endocrine system during early development makes them vulnerable to anthropogenic sources of EDCs that invariably end up in aquatic systems. One such source of EDCs of interest is run-off from agricultural fields amended with poultry litter as fertilizer. The Delmarva Peninsula, occupied by portions of three states: Delaware, Maryland and Virginia, is one of the largest poultry producing regions in the United States (USDA 2002). Due to the large expansion of the poultry industry over the last 25 years, poultry operations have become high production rate facilities or CAFOs (concentrated animal feed operations). The Delmarva poultry farms produce more than 600 million birds annually and generate 15% of the Chesapeake Bay region's total livestock manure by weight (Chesapeake Bay Foundation 2004). As manure is generated, it is collected and stored until it becomes designated as cropland fertilizer. Because transporting manure over large distances is difficult and not cost-effective, farmers usually spread manure on fields close to where it was produced. Constant application to nearby areas can lead to an excessive loading of nutrients and other compounds which are then more susceptible to run off (Chesapeake Bay Foundation 2004).

The accumulation of steroid hormones in the urine and feces of livestock is a naturally occurring process. However, coupled with the large accumulation of manure over short periods of time, the high concentrations of steroid hormones in poultry litter applied as fertilizer are a potential source of EDCs. Specific metabolites that may be found in livestock manure will differ according to species, sex, and class of farm animal (Hanselman et al. 2003). The primary estrogens excreted by poultry are 17β-estradiol,

estrone and estriol whereas the primary androgens are testosterone and dihydrotestosterone (Shore et al. 1993; Moore et al. 1982). Steroid hormones in animal feed-lot effluent and agricultural run-off are subject to metabolic conjugation prior to excretion by livestock, depending on the species (Hanselman et al. 2003). Poultry excrete estrogens (etradiol, estriol and estrone) as conjugates or in the non-conjugated forms in both urine and feces; (Hanselman et al. 2003). The physicochemical properties of androgens and estrogens dictate their behavior in sediments or water. Both androgens and estrogens are tetracyclic molecules, and key functional groups located at the C-3, C-16 and C-17 positions will determine their activity and binding to receptor sites (Hanselman et al. 2003). When steroid compounds are utilized physiologically and then excreted into the urine of an organism, they become conjugated with the addition of a sulfate group or glucuronide group at the C-3 and/or C-17 positions to make them more water soluble and hence easier to excrete (Hanselman et al. 2003). Steroidal compounds excreted into the feces are unconjugated and therefore may become more bioavailable in sediment or water (Khanal et al. 2006). Free estrogens are hydrophobic with log K_{ow} values ranging from 2.6 to 4.0 indicating that sorption to solids is high and reported values for organic-carbon sorption coefficients or log K_{oc} values (~2.8-3.8) are consistent with solubility values (Das et al. 2004; Lai et al. 2002). Several studies have found that the biological activity of estrogens in soils in particular is greatly reduced within several hours or days due to microbial activity (Das et al. 2003). Similarly with androgens such as testosterone, factors affecting the concentration in poultry litter are reliant upon the amount of time the litter is allowed to sit until it is applied as a soil amendment (Shore et al. 1993). Livestock wastes therefore are a potential source of endocrine disrupting compounds (Hanselman et

al 2003). Steroidal estrogens and androgens are of particular concern in poultry litter because they can persist in low, nanogram per liter concentrations, which are biologically relevant and thus may adversely impact the reproductive biology of fish and other aquatic species (Hanselman et al. 2003). Chronic lifetime exposure is likely for resident fish populations that do not migrate greater than 50 m from their natal areas such as the mummichog (*Fundulus heteroclitus*), sheepshead minnow (*Cyprinodon variegatus*) and the fathead minnow (*Pimephales promelas*) (Boudreau et al. 2004; Hanselman et al. 2003; Zillioux et al. 2001; Ackerman and Taylor 1995).

1.3 The fathead minnow model

The fathead minnow was chosen as the model species for this study for several reasons. First, fathead minnow are found in areas surrounding the Chesapeake Bay in farm ponds and retention ponds (MBSS 2008). The use of poultry litter as fertilizer often creates an over abundance of litter entering water systems, making the fathead minnow, among other related species, vulnerable to EDCs in run-off (MBSS; Chesapeake Bay Foundation 2004). Second, the reproductive physiology of the fathead minnow is well known (Harries et al. 2000; Ankley et al. 2001; and Jensen et al. 2001). Reproductively mature male fathead minnow have several secondary sex characteristics under control of circulating hormones, making these traits susceptible to EDC exposure. Development of these essential male characteristics has been shown to become compromised with exposure to an exogenous source of EDCs (Ankley et al. 2004; Bringolf et al. 2004; Ankley et al. 2003; Länge et al. 2001; Harries et al. 2000).

The relationship between expression of secondary sex characteristics and levels of circulating hormones is often complex. Feedback mechanisms inherent within the

physiological framework of the reproductive axis must be considered (Ankley and Johnson 2004). Furthermore, the expression may be affected by the ability of the organism to maintain homeostasis of endogenous hormone circulation as well as by the levels of EDCs that may be present in the poultry litter itself (Yonkos 2005; Hanselman et al. 2003). Thus, my objectives were 1) to characterize the content of a poultry litter mixture in terms of steroid hormone concentrations: testosterone and 17β-estradiol, 2) to determine how poultry litter as a source of EDCs can alter the development of gonadal tissue and subsequently affect gender ratio in adult fish if exposure occurs during the larval stage, 3) to measure the impact of poultry litter exposure at the larval stage on the expression of male secondary sex characteristics at the adult stage, and 4) to determine if aromatase activity becomes altered by poultry litter exposure during sexual differentiation.

2. Materials and Methods

2.1 Chemical analysis of poultry litter

For steroid hormone analyses, the poultry litter leachate was prepared with different stir times, incubation times, deconjugating enzymes, or spiked with known concentrations of steroid hormones (Table 2.1). The leachate was analyzed with a radioimmunoassay (RIA) for steroid hormones using methods that have been previously described (McMaster et al. 1992). Briefly, prepared samples were run in duplicate then frozen at -20 °C until analysis. Antibody was diluted 1:70 for 48% binding prior to adding to each sample. Samples were then incubated at 4° C with dextran-coated charcoal for 24 hr, then centrifuged at 3,000 rpm. The supernatant was decanted and added to scintillation vials with 5 mL of scintillation cocktail (Perkin-Elmer, Waltham, MA) and

counted in a scintillation counter measuring counts per minute (CPM) for five minutes per vial (McMaster et al. 1992). Polyclonal antibodies for estradiol and testosterone were purchased from Accurate Chemical and Scientific Corporation (Westbury, NY). Samples were prepared prior to RIA analysis as described below.

Poultry litter was stirred either long-term (24 h) or short term (4 h) to determine if stirring duration impacted detection of steroid hormones in the leachate. Samples were either incubated at 37 °C for 1 hr and then frozen at -20 °C until analysis, or prepared and then immediately frozen at -20 °C until further analysis. This preparation step was to determine how increased temperature would affect the availability of the steroid hormone content in the litter. Spiking samples of the PLL with a known amount of testosterone (T) or estradiol (E2) was used to validate the RIA method. A stock solution of steroid hormone was prepared by dissolving 10 mg of either T or E2 in 30 mL of ethanol. This solution was then dissolved in 100 mL of deionized H_2O. From this primary stock, 5 µL was then added to 5 mL of PLL to create a 40 ng/L concentration of steroid hormone. To evaluate levels of glucuronide and sulfate conjugation products in the leachate, a subset of samples were treated with β-glucuronidase (250 units/ml) and aryl-sulfatase (5 units/ml; Boehringer-Mannheim Biochemicals, Indianapolis, IN; Payne and Talalay 1986). A summary of these treatments is shown in Table 2.1.

2.2 Fish exposure to poultry litter experiment

All animal procedures were performed under approved Institutional Animal Care and Use Protocols at the University of Maryland, College Park. The experiment was performed in May of 2005. Larval fathead minnow, 0-3 dph (days post-hatch) were randomly selected and placed into one of six treatments in a static exposure vessel (n=65

larvae/vessel). For each of six treatments, two exposure vessels were designated making a total of twelve tanks and 130 larvae per treatment. Each exposure vessel was a 4000 mL beaker filled to 2000 mL and equipped with an airstone to provide gentle aeration and circulation of the water. All animals were offspring of breeding groups from a population maintained at the Aquatic Pathobiology Center, College Park, MD. Animals were maintained at 25±2°C and a 16:8 light:dark photoperiod for the duration of the study. Larvae were fed live artemia twice daily for thirty days and thereafter were fed a combination of flake food (Tetramin®, Tetra,Co.) and slow-sinking pelleted crumble diet (Finfish Starter #2, Ziegler Bros., Inc. Gardener, PA), until sacrifice at eight months of age.

2.3 Poultry litter preparation

Poultry litter was randomly collected from a broiler operation on the Eastern Shore of Maryland in Fall of 2004. Litter was mixed, then hammer-milled, and mixed again to generate homogeneous batches of particulate material. A stock concentration of poultry litter was prepared every other day, using carbon-filtered tap water, for thirty days as a 0.25% weight by volume solution (Nichols et al. 1997). Litter was suspended in carbon-filtered tap water at room temperature for 4 h and then coarse filtered through Whatman #1 filter paper on a Buchner funnel. Water was drawn by vacuum and the filtrate was diluted to make the 1:4, 1:8 and 1:16 fold dilutions of poultry litter leachate (PLL) for three exposure treatments. These dilutions were based on previous research that showed environmentally relevant and biologically active levels of steroid hormone within the PLL as well as water quality conditions as tolerable for fathead minnow (Yonkos 2005; Ankley et al. 2001; Diamond et al. 1993; U.S. EPA 1987).

2.4 Experimental treatments and procedures

Treatments consisted of a negative control (0 g/L PLL), solvent control containing 0.001% ethanol, a positive control (100 ng/L 17β-estradiol), and the three dilutions of litter extract as described above. The three dilutions were 1:4, 1:8, and 1:16 of a stock PLL preparation, contained 0.625 g/L, 0.3125 g/L and 0.15625 g/L, respectively. All fish were exposed to the treatments for thirty days in 4000 mL beakers and then removed and placed into 9.5 L tanks (filled to capacity) for the grow-out phase of the study. After sixty days in the 9.5 L tanks, all animals were removed and placed into 21 L tanks (filled to capacity) until sacrifice at eight months of age.

2.5 Positive control preparation

The positive control of 17β-estradiol (E2) was prepared by dissolving 40 mg of E2 in 1 mL of ethanol. This solution was then diluted 1:100,000 with ethanol yielding a final concentration of 4000 ng/mL. The E2 solution, in a volume of 100 μL was added to each of the two replicates for the positive control treatment to make a final concentration of 100 ng/L.

2.6 Water quality

Throughout the experiment, water quality was measured every other day, once before a water change and once after each water change for thirty days. After thirty days, water quality was measured once a week. Water quality parameters recorded were temperature, NH_3, NO_2, NO_3, and pH. All exposure and grow-out vessels were cleaned each day to remove particulate matter and any uneaten feed to maintain water quality.

2.7 Fish analyses

All fish were euthanized at eight months using an overdose of buffered MS-222 (300 mg/L buffered with 200 mg $NaHCO_3$/L) and body weights and lengths were measured to determine condition factor (body weight (g)/ length $(mm)^3$) X 100). Brain tissue was removed and weighed to the nearest 0.001 g, then placed into 100 µL of a potassium phosphate buffer (pH 7.4), homogenized for 20 s using a Fisher® Powergen 125 homogenizer and then placed at -80°C until further analysis for aromatase activity. Aromatase activity was analyzed using a method modified from Thompson and Siiteri (1974) and reported per mg of protein. A 3 µL aliquot of homogenate was also set aside and diluted 1:10 and performed in duplicate on all brain tissue using a BCA Protein Assay Reagent Kit (Pierce Chemicals, Dallas, TX). Protein samples were prepared in a 96-well microtiter plate and analyzed at 570 nm on a spectrophotometric plate reader. Aromatase activity is calculated as femtamoles per hour and then mg of protein are determined to standardize per brain homogenate. Aromatase activity is reported as femtamoles per hour per mg of protein.

The morphometrics in male fathead minnow that were assessed included male breeding tubercle counts and male dorsal epithelial pad presence (Harries et al. 2000; Smith and Murphy 1974). Breeding tubercles were counted under a dissecting scope. Epithelial pad presence was ranked by absence or presence of an epithelial pad according to methods previously described (Smith and Murphy 1974). All fish were dissected laterally at the head and caudal peduncle (just at end of gut package) and body segments were preserved in 10% buffered formalin for histological analysis.

Histological preparation and analysis (staging) of the gonads was conducted according to Ankley et al. (2001). For histological preparation, animals were divided into "small" (28-38 mm length) or "large" (39-50 mm length) and were processed accordingly. For "small" fish: four, 5μm sections were prepared with 600 μm between each section. For "large" fish: four, 5 μm sections were prepared with 1000 μm between sections. Evaluation of the testes was based upon the degree of spermatogenic cell types whereas ovaries were assessed based upon relative stage of oocyte maturation (Ankley et al. 2003; Ankley et al. 2001; Harries et al. 2000; McDonald et al. 2000). Gonadal cell types were staged and then ranked, for male and female fathead minnow using the following criteria, modified from methods previously described (McDonald et al. 2000). For ovaries, scores were 0=immature (undeveloped, no distinguishable oocytes), 1=early development (oocytes pre- to early vitellogenic), 2= mid-development (mid-vitellogenic oocytes), and 3=mature (mature, late vitellogenic oocytes). For testes, scores were 0=immature (undeveloped, no distinguishable sperm cell types), 1=early spermatogenic (spermatogonia and spermatocytes observed), 2= mid-spermatogenic (increased number of spermatogonia, spermatocytes and spermatids), and 3=late spermatogenic (mature spermatozoa, all stages of development are represented).

2.8 Statistical analyses

All data for chemical analysis of the PLL preparations were analyzed using the Student's t-test. Comparisons were made between the short stir time (4 hr) v. long stir time (24 hr), incubation at 37°C short stir time (4 hr) v. incubation at 37°C long stir time (24 hr), short stir time (4 hr) with added deconjugating enzymes v. long stir time (24 hr) with added deconjugating enzymes, and short stir time (4 hr) with recovery method v.

long stir time (24 hr) with recovery method. Aromatase data, condition factor, and male tubercle count data were analyzed for differences among treatments using analysis of variance followed by Tukey's test for mean separation. If data did not meet assumptions for ANOVA (i.e., normal distribution of data), the appropriate transformation was made (square root transformation for condition factor and tubercle count) and data are presented as back-transformed. The Fisher's Exact test was used to analyze epithelial pad presence in males, gonadal cell stage, and gender ratio. All statistical analyses were performed using SAS® 9.1 (Cary, N.C.) and evaluated at $\alpha = 0.05$ level of significance.

3. Results

3.1 Chemical analyses of poultry litter

Chemical analyses of the PLL showed detectable levels of testosterone and estradiol in all samples (Table 2.2). Recoveries for the RIA analyses were > 89.1% and the sensitivity for the E2 RIA was determined to be 60 pg/mL and 20 pg/mL for the testosterone assay. Comparisons were made between poultry litter preparations to determine if changes in stir time, addition of the conjugating enzymes or heating of the sample would alter the steroid concentrations detectable. The length of time for stirring of the poultry litter (long term; 24h, versus short term; 4h) had no significant effect on testosterone ($p=0.99$) but did significantly affect estradiol in the poultry litter mixture ($p=0.03$). Samples spiked with either E2 or T (40 ng/L) did not alter estradiol ($p=0.06$) or testosterone ($p=0.94$) content of the litter demonstrating the accuracy of the RIA method. An increase in approximately 40 ng/L for each sample was observed.

Heating the sample in conjunction with a long stir time or a short stir time did not alter estradiol detection ($p=0.98$) but significantly affected testosterone ($p=0.0003$).

Addition of deconjugating enzymes β-glucuronidase and aryl-sulfatase had no effect on the levels of estradiol detected in the sample ($p=0.84$) but did significantly increase the amount of testosterone detected in the sample ($p=0.01$).

3.2 Fish exposed to poultry litter

All water quality parameters fell within acceptable levels for maintaining fathead minnow in aquaria (U.S. EPA 1987). Mortalities occurred for the majority of the exposure period during the first month. Survival was ~65% for all PLL groups at 30 dph, and >85% for all other groups at 30 dph. Mortalities occurred throughout the exposure period and until sacrifice. However, these later mortalities were fewer in frequency, fewer in number, and most likely not treatment related (delayed mortality from the first month of exposure). Instead, the later mortalities were most likely due to high stocking densities because they occurred in all exposure vessels from 2 months to 8 months. High mortality (~60%) for fish at this stage of development is typical when stocking densities are high (>300 larvae/L in 22 L vessels) (Baskerville-Bridges and Kling 1999). My fish were contained in 4 L vessels filled to 2 L at 65 fish for the first 30 days of their lives which is 5 times the acceptable density for larvae at this stage, most likely causing the high mortality observed in my study (approximately 56% mortality) in the first two months after depuration, even though fish were moved to larger containers thereafter (9.4 L and then 21 L). The stocking densities in my study were maintained at high numbers initially to sort out the fittest individuals per treatment. The greatest amount of mortaility naturally occurs during the early life stage of development of fishes and results in selecting out individuals that may not survive otherwise (Houde 1997). It is also well established that body size and temperature are the two variables that have most clearly

demonstrated to be related to survival and productivity during early development (Houde 1997). This theory also translates well to microcosm studies as high stocking density should not interfere with normal growth patterns. Shaw et al (1995) states that as high numbers of larval fathead minnow in microcosms were larger in number and resulted in smaller fish size, this large biomass did not alter development and therefore growth was found to be not related to density dependent effects. In addition, compensatory growth occurs in larvae that start out as smaller individuals once they get past the initial selective phase (Springate and Fromage 1975).

Both male and female fish were included in the condition factor analysis (data not shown). Results showed no significant differences among treatments ($F=1.16$; $df=5, 144$; $p=0.19$. Aromatase activity did not differ between treatments within gender ($p=0.47$; Figure 2.1). However, differences between males and females were significant with respect to aromatase activity ($p=0.03$). Males exhibited a 24% significantly lower aromatase activity compared to females ($F=1.04$; $df=5, 136$; $p=0.03$).

A significant difference was found among treatment groups for numbers of breeding tubercles in males (Figure 2.2; $p=0.02$). The 1:8 treatment group had a significantly higher number of breeding tubercles than the control ($p=0.04$), the 1:4 ($p=0.03$), the 1:16 ($p=0.02$), the estradiol treatment group ($p=0.0003$), and the solvent control ($F=3.10$; $df=5, 51$; $p=0.004$).

Similarly, the percent presence of a dorsal epithelial pad followed the pattern of breeding tubercle number (Figure 2.3). A significant increase in dorsal pad presence was observed in the 1:8 PLL concentration where 100% of the males had developed a pad ($p=0.05$). Compared to controls, 47% more males in the 1:8 treatment had a dorsal

epithelial pad. Also, 50% more males in the 1:8 treatment had dorsal pads than males in the solvent control, 1:4, and 1:16 treatments. Compared to the males in the estradiol positive control group, 78% more males in the 1:8 treatment had dorsal pads compared to males in the positive control group ($p=0.02$; $F=1.66$; $df=5, 51$).

No significant differences were observed with respect to percent difference of gonadal cell types in the ovaries of female fish ($p=0.34$; Figure 2.4). However, females did exhibit more variability with respect to the maturity of individual ovarian cells indicating what would be expected in a developing ovary. In males, a significant difference was found in the percent of gonadal cell types among treatments ($p=0.02$; Figure 2.5). The 1:4 PLL treatment group had a significantly lower number of immature testes as compared to mature testes than the control ($p=0.05$).

Analysis of adult gender ratio indicated that there was a significant difference where females outnumbered males ($p=0.02$; Figure 2.6). Specifically, treatments with a greater number of females compared to males, were the 1:4 ($p=0.04$), and the 1:8 ($p=0.009$). No significant differences were found between the control group and the 1:16 PLL treatment ($p=1.0$) nor between the solvent control and the estradiol positive control treatment group ($p=0.80$; $F=2.45$, $df=5, 136$).

4. Discussion

The Chesapeake Bay has more land which drains run-off into it than any other bay in the world (Chesapeake Bay Foundation 2004). Heavy rains and high overland flow make the Chesapeake very vulnerable to pollutants that come off the land, particularly in agricultural regions. Run-off from agricultural lands is a significant source of contaminant loading because of the use of manure as fertilizer and because of the lack of

storage capacity for the over abundance of the manure that is produced (Chesapeake Bay Foundation 2004).

The heavy use of poultry litter as fertilizer in this region has the potential for endocrine disrupting effects especially when animals are exposed during labile, sensitive stages of early development. The biology and reproductive endocrinology of the fathead minnow has been studied extensively and therefore, the exposure period for my study was the known period of sexual differentiation in these animals (0-20 dph; Ankley et al. 2003; van Aerle et al. 2002; Ankley et al. 2001; Jensen et al. 2001).

Field surveys of soils, surface, and ground waters have demonstrated both detectable and environmentally significant levels of steroid hormones. Variation in reported levels of steroid hormones in soil and water samples exist due to difficulties related to analyzing complex matrices like a leachate (Lee et al. 2003). Low solubility in water (0.8-13.3 mg/L; Hanselman et al. 2003) and hydrophobicity render steroid compounds highly stable, highly microbial resistant, and bioavailable to aquatic organisms (Hanselman et al. 2003). The physicochemical properties of steroids are also such that they have high binding affinities to substrates like organic matter and subsequently, plastics or glassware used in analytical laboratory procedures (Das et al. 2003; Hanselman et al. 2003; Lee et al. 2003; Lai et al. 2002). As such, I prepared the leachate in a variety of ways to aid in coaxing the steroids into solution so they might be more easily detected by the methods used and subsequently get a better estimate of the levels of steroid hormone to which the larval fish may be exposed.

My study demonstrated relatively high levels, in terms of what is biologically active, of both testosterone and estradiol found in the poultry litter leachate (Table 2.2)

and these values fall within the range for what has been measured in manure impacted waters in the field for estradiol and testosterone using RIA methods (23 ng/L to 3500 ng/L in streams, ponds and run-off waters; Hanselman et al. 2003; Nichols et al. 1997; Shore et al. 1995). ELISA methods prove similarly sensitive (run-off waters from poultry litter were approximately 150 ng/L; Finlay-Moore et al. 2000). Concentrations of steroid hormones will vary among poultry litter samples depending on many factors including the method of application of the litter, rate of application of the litter, and rain events (Nichols et al. 1997). It is also probable that with the current detection methods, it may be difficult to obtain a consistent measure of the steroids due to interference by other compounds in the matrix. Previously, tested clean-up methods (an alumina column; aluminum oxide, a carbograph column, or a C-18 solid-phase extraction column) proved unable to remove unwanted compounds completely while simultaneously keeping the steroid hormones in the leachate mixture (unpublished data).

I found that levels of detectable testosterone and 17β-estradiol in the poultry litter leachate were several fold of endogenous levels found in plasma of fathead minnow (Jensen et al. 2001). I also found that when exposed during the larval stage to environmentally relevant concentrations of this poultry litter leachate, fathead minnow exhibited a skewed gender ratio toward a majority female population. The resulting impact of this higher female to male ratio caused the small percentage of males to develop male secondary sexual characteristics such as breeding tubercles and dorsal epithelial pads. While this may be beneficial to increase competition among males in a given area, fewer numbers of males can result in less genetic information into subsequent generations.

No adverse effects on aromatase activity were observed in my experiment. Other studies have shown that exogenous exposure during the period of sexual differentiation to steroid hormone mimics can alter aromatase expression and skew gender ratio (Fenske and Segner 2004; Scholz and Gutzeit 2000). In zebrafish, 35-40 dph, exposure to fadrozole or 17α-methyltestosterone resulted in altered expression of the CYP19 gene mRNAs and an all male population of zebrafish (*Danio rerio*), even after the treatments were removed after one month of exposure (Fenske and Segner 2004). In my study, exposure to the PLL may have altered the aromatase activity in individuals during a period of sexual differentiation, thereby implementing effects on processes further along the reproductive axis (i.e., steroid hormone production, sexual differentiation, and feed-back). However, once animals were removed from the exposure, the individuals that may have experienced alterations in these down stream effects did not demonstrate indications of alteration of the aromatase enzyme once the animal reached the adult stage. This is supported by evidence that aromatase activity levels were within the range for what is expected for differences between genders (Fenske and Segner 2004; Melo and Ramsdell 2001). Orlando et al. (2002) showed that although masculinization of females occurred with exposure to paper mill effluent in the mosquitofish (*Gambusia holbrooki*), the change in aromatase was not the mechanism and may instead be explained by feed-back mechanisms along the HPG axis. Therefore, the observed phenotypic gender of an organism can differ from the cell type within the gonadal tissues. This will be further discussed in the section describing gonad cell stage effects.

Male fathead minnow have a number of secondary sexual characteristics under direct hormonal control. Alterations in the presence of the epithelial pad and changes in

breeding tubercle number have been linked to EDC exposure (Pawlowski et al. 2004; Ankley et al. 2003; Ankley et al. 2002; Parrott and Wood 2002; Zerulla et al. 2002; Länge et al. 2001; Harries et al. 2000; Miles-Richardson et al. 1999; Nichols et al. 1999). I found that larval exposure to poultry litter leachate caused the males in the 1:8 PLL mixture to have the greatest number of breeding tubercles.

Previous studies have shown that tubercle count is a more robust measure of endocrine disruption as compared to measuring changes in the epithelial pad, and pad development may not respond as well to circulating androgens (Panter et al. 2004; Parrot and Wood 2002; Hemming et al. 2001; Länge et al. 2001; Smith 1974). Yet, I observed that the presence of the epithelial pad and number of breeding tubercles was highest for males for the same intermediate level of the PLL (1:8 concentration; Figure 2.3). Among fathead minnow populations, males will develop hierarchies based on dominance and acquisition of nesting territories (Martinović et al. 2007; Danylchuk and Tonn 2001). Development of secondary sexual characteristics such as the epithelial pad and breeding tubercles normally occur only in more dominant individuals in the population (Martinović et al. 2007; McMillan and Smith 1974). With fewer males in the population of the 1:8 poultry litter treatment (N=5 males and N=20 females), competition among males might have escalated to the extent that tubercle growth and epithelial pad development occurred among all males. With few males in a population, size discrimination among males is more difficult to ascertain for individuals (Danylchuk and Tonn 2001).

Gonadal cell development is critical to maintenance of proper signaling and feed-back activity of steroid hormones, pituitary gonadotropins, and GnRH from the

hypothalamus, and along the reproductive axis (Devlin and Nagahama 2002). Poultry litter run-off may impact gonadal cell differentiation if exposure to steroid hormones or steroid-like compounds occurs during critical windows of development. Exposure during this stage of development can also result in complete phenotypic sex reversal and skewed gender ratio (Scholz and Gutzeit 2000). Altered gender ratios within a population can have profound effects on subsequent generations (Roex et al. 2001).

I observed no differences in gonadal cell types among females for the different treatments. However, in males, there was a significant difference between treatments for gonadal stage. I observed varying stages of gonadal development for both genders and mature testes were observed more often than immature except in the case of the estradiol positive control. The observation of fewer mature testicular cell types is similar to what has been observed in other studies when fish are exposed to estrogen agonists (Bringolf et al. 2004; Nash et al. 2004; Miles-Richardson et al. 1999). Male fish were present in the positive control (E2) treatment, yet very few males were reproductively mature based on testes cell differentiation. Previous studies have shown that with exposure to estradiol, females tend to show greater variability in gonadal staging of cells and males tend to demonstrate fewer mature testes (Fenske et al. 2005; Seki et al. 2005; Pawlowski et al. 2004; Nichols et al. 1999).

Males had a greater amount of mature testes (aside from the positive control) in the intermediate level (1:8 concentration) of the poultry litter leachate exposure. This may be because while estradiol is present in the PLL, it may only be present at sufficient levels in the PLL to alter the gender of some males, but not able to diminish testicular maturation in remaining males as supported by Orlando et al. 2002; Parrot and Wood

2002; Hemming et al. 2001. In addition, because all males were mature in these treatments (displaying breeding tubercles and dorsal epithelial pads), higher levels of circulating androgen may have enhanced the mature stage of the males and caused increased competition among them (Harries et al. 2000; Ankley et al. 2001; Jensen et al. 2001).

Alteration of gender ratios within a population can stem from the altered development of gonadal tissue (Blázquez et al. 1998). I found gender ratio in the highest PLL (1:4) and intermediate PLL (1:8) treatments was skewed toward female. Given that the survival percentages were considerably low, the question of whether or not the gender skewing may be related to differential survival among genders is a possibility. However, this theory is not supported based on the following evidence and instead the gender skewing is more likely related to the poultry litter exposure: When fathead minnow larvae develop, smaller larvae usually do not survive as well as larger larvae. As they develop, gender is determined and a larger percentage of the larvae usually turn out to be male. This indicates that the male larvae, although not phenotypically differentiated, are larger during this stage and have more of a survival advantage (Norman Sinitski, ARO, personal communication). In terms of survival results for larval fathead minnow, this indicates that the poultry litter effect is more likely an explanation for the gender skewing observed because if it were not, then it would be that male larvae would have a survival advantage and I would have observed the opposite gender skewing, toward male.

With respect to resulting feminization or masculinization of fish populations exposed to EDC mixtures, Parrott and Wood (2002) reported both a masculinization of female fathead minnow (presence of breeding tubercles and lateral band pattern) and

feminization of male fathead minnow (development of an ovipositor) exposed to bleached-sulphite mill effluent (BSME). My study showed similar effects from exposure to the PLL. Gender ratio was female biased and no feminization of males occurred in terms of secondary sex characteristics; however, male secondary sexual characteristics were more pronounced in the more concentrated PLL treatments. Despite the lack of significant skewing of the gender ratio toward female in the positive control treatment group, the ratio of females to males in this treatment group was greater than an expected 50:50. The amount of estradiol required to observe changes in secondary sex characteristics is greater than what is required to cause changes in gonadal cell differentiation or reproductive physiology (Ankley et al. 2002). Therefore, the concentration of E2 within the test chambers may have been lower than nominal and without verification of the concentrations; I can not draw any conclusions based on the E2 positive control group. In addition, only the more concentrated PLL treatments in this study may have had sufficient levels of E2 to cause these changes and not the positive control group.

To my knowledge, this is the first study to investigate the response from an EDC mixture such as a poultry litter leachate on larval fish and then to conduct observations on these animals at maturity. The importance of my findings is reflected in the fact that male characters and gender ratio were significantly impacted as manifested at the adult stage, despite a depuration step for 7 months with no PLL exposure. These results illustrate the fact that larval fish are extremely vulnerable to exogenous exposure of mixtures containing potential EDCs. Because the timing and duration of exposure can permanently alter development, the manifestations of these effects may not be visible until adulthood;

when proper hormone signaling for reproductive events can translate to survival for a given population.

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Table 2.1. Poultry litter leachate sample preparation prior to RIA analysis. Each sample preparation was in duplicate and a total volume of 5 mL of leachate.

1) Incubate at 37 °C (short-term stir of PLL: 4 h) β-glucuronidase +aryl sulfatase,	**2) β-glucuronidase +aryl sulfatase only, no litter (water control)**
3) Incubate at 37 °C, β-glucuronidase + aryl sulfatase (long-term stir of PLL: 24 h)	**4) Incubate at 37 °C(short-term stir of PLL: 4 h)**
5) spike w/T and E2 (short-term stir of PLL: 4 h)	**6) short-term PL (4 h), no prep**
7) long-term stir of PLL (24 h), no prep	**8) Incubate at 37 °C(long-term stir of PLL: 24 h)**

Table 2.2. 17β-Estradiol and testosterone concentrations in poultry litter leachate. Samples were in replicate and measured using a RIA method. Values are means ± SEM. MDL = 18 pg/mL; LOQ = 20 pg/mL.

Sample Preparation	17β-Estradiol (pg/mL)	Testosterone (pg/mL)
short-term stir of leachate (4 h), no preparation	290.7 ± 6.100	196.2 ± 5.600
long-term stir of leachate (24 h), no preparation	359.8 ± 18.20	102.6 ± 11.90
Incubate at 37 °C (short-term stir)	301.3 ± 6.700	243.9 ± 4.000
Incubate at 37 °C (long-term stir)	403.7 ± 21.30	102.7 ± 8.400
spike w/T and E2 (short-term stir)	319.8 ± 9.8	224.8 ± 2.800
β-glucuronidase +aryl sulfatase, Incubated at 37 °C (short-term stir)	387.6 ± 44.80	401.6 ± 6.000
β-glucuronidase + aryl sulfatase, Incubated at 37 °C, (long-term stir)	455.3 ± 25.10	164.9 ± 2.000
β-glucuronidase + aryl sulfatase, no litter (water control)	MDL	LOQ

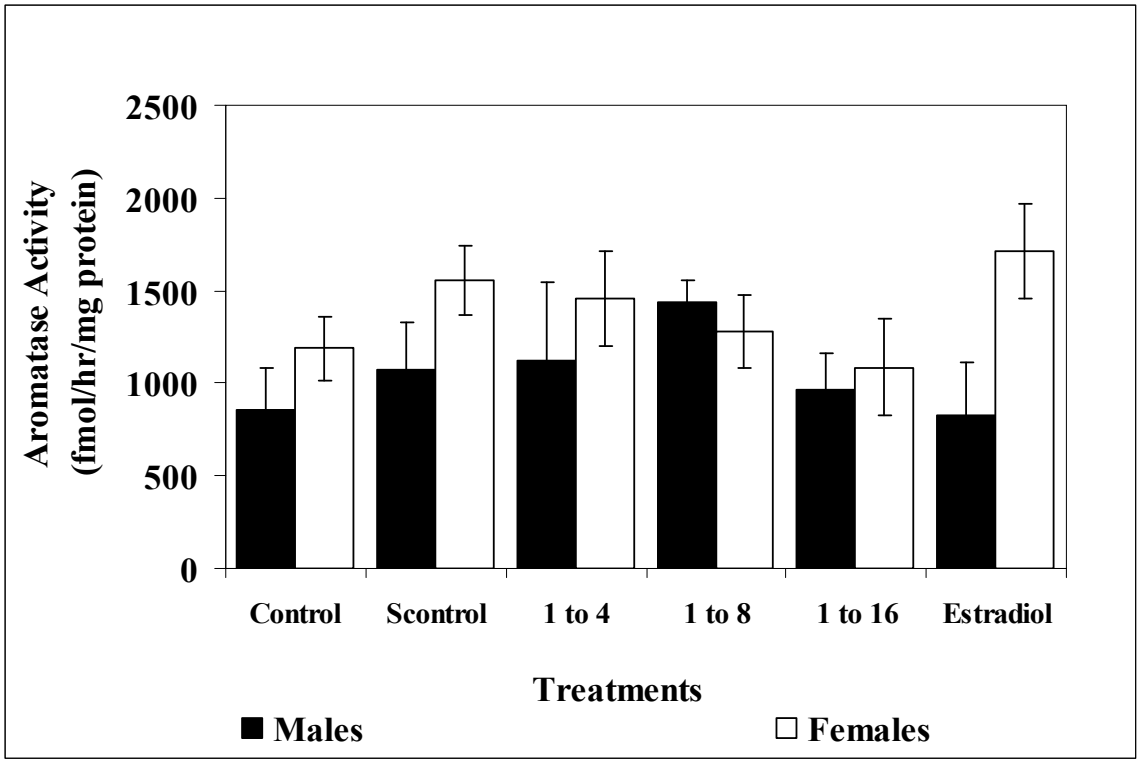

Figure 2.1. Aromatase activity in male and female fathead minnow. Males expressed a significantly lower aromatase activity as compared to females (p=0.03). Data are means ± SEM; N=21-30 animals/treatment.

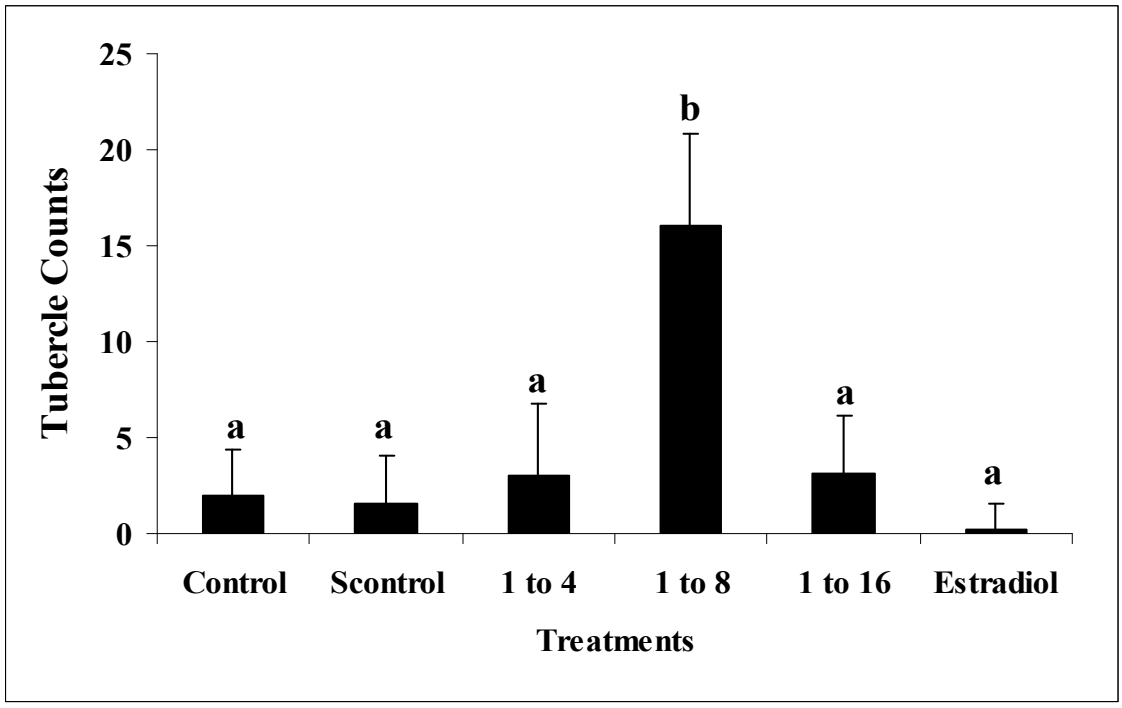

Figure 2.2. Tubercle counts in male fathead minnow. Differences between treatments are indicated with different letters. N=6-15 males/treatment (p=0.02).

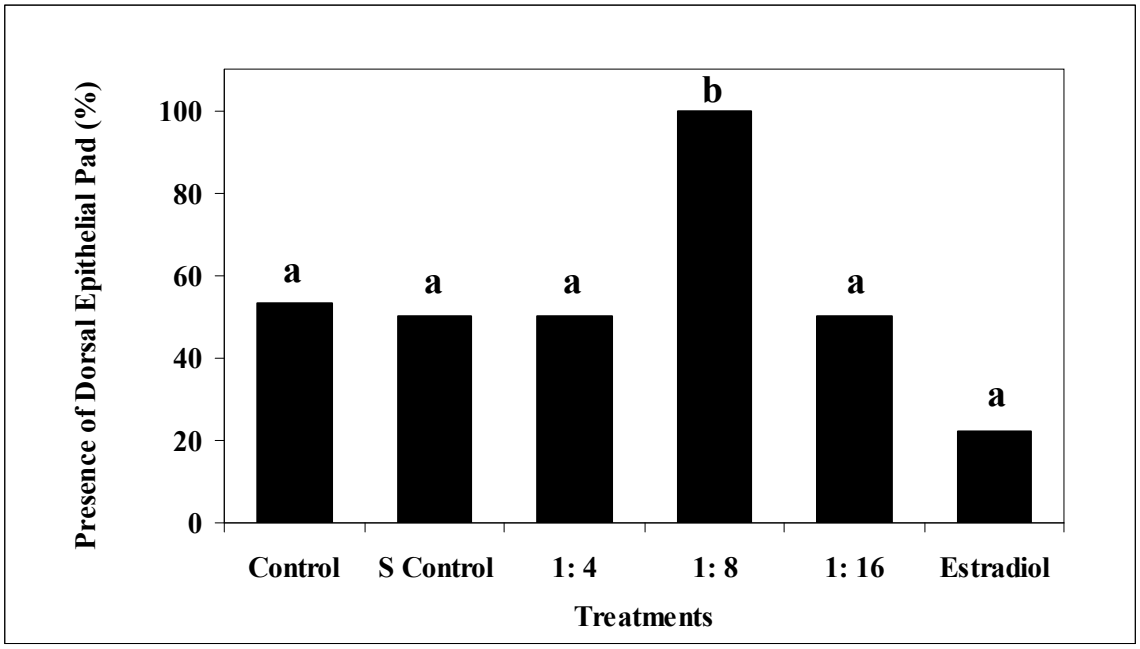

Figure 2.3. Dorsal epithelial pad presence in male fathead minnow. Differences are indicated by different letters. Values are percent presence of an epithelial pad; N=6-15 males/treatment.

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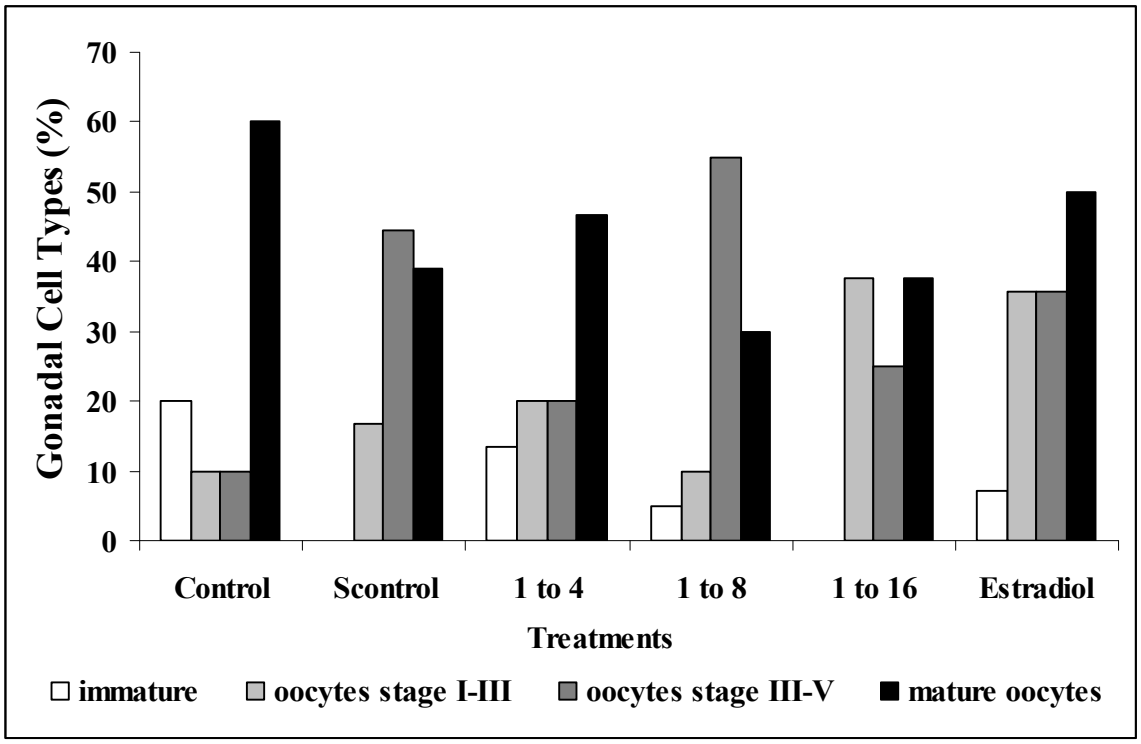

Figure 2.4. Gonadal cell type percentages for female ovarian tissue. Values are percent of each ranked gonadal stage of development (N=8-20 females/treatment; p=0.35).

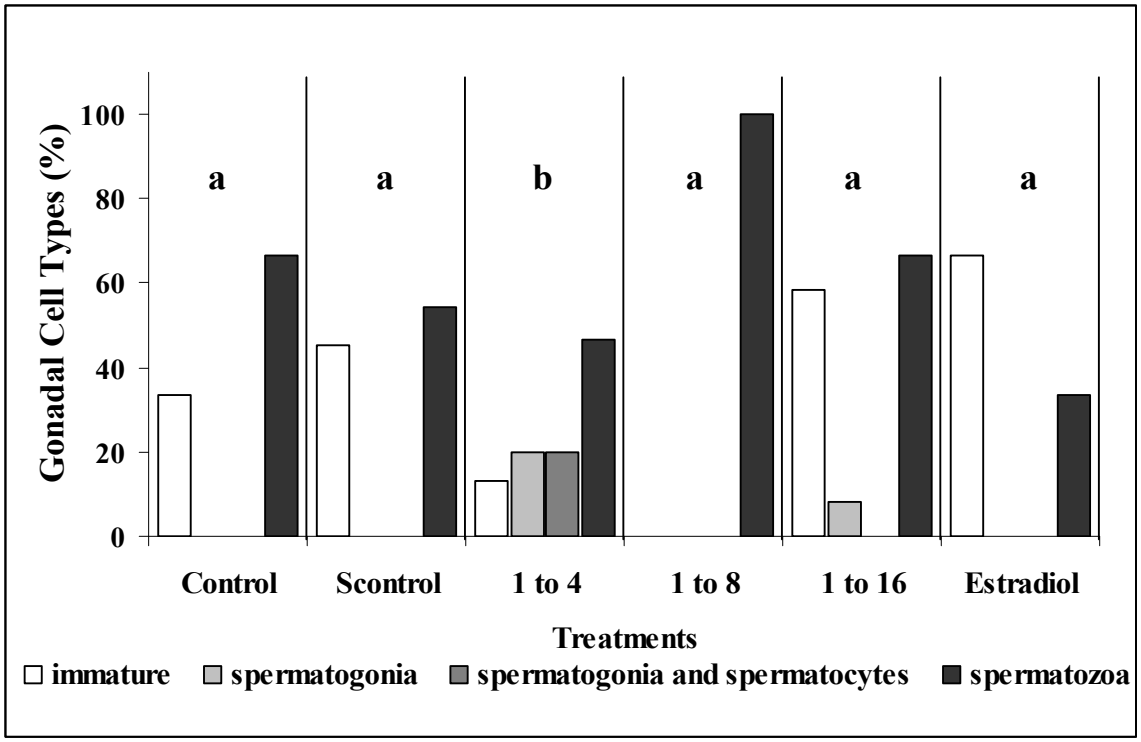

Figure 2.5. Gonadal cell type percentages for male testicular tissue. Different letters indicate differences with respect to different percent cell types. Values are percent of each ranked gonadal stage of development (N=8-20 males/treatment; p=0.05).

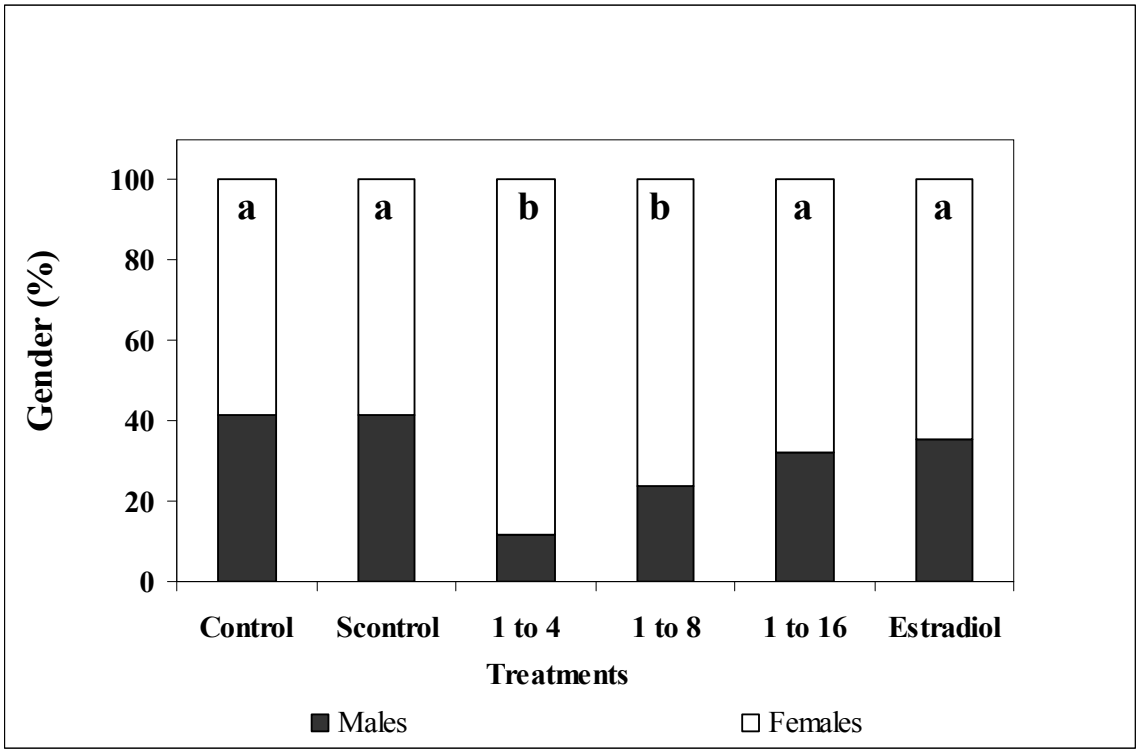

Figure 2.6. Gender ratio of fathead minnow expressed in terms of percent male or percent female in each treatment group. Treatments that differed significantly from an expected 50:50 gender ratio are represented with different letters; N=21-30 fish per treatment.

Chapter 3: Transgenerational Reproductive and Developmental Response to Poultry Litter Exposure in the Fathead Minnow (*Pimephales promelas*)

Abstract

The overuse of poultry litter as a fertilizer is of regional concern because agricultural run-off carrying unknown contaminant mixtures enter aquatic systems that feed into the Chesapeake Bay. The contaminants or potential endocrine disrupting compounds (EDCs) may target the endocrine system in fish, disrupting their physiology, behavior, and reproductive success. To examine the effects of poultry litter on a model fish, the fathead minnow, *Pimephales promelas*, I exposed adult fish and their offspring to varying levels of poultry litter leachate. Adults (P_1 Generation) were exposed to two different concentrations of a poultry litter leachate (High PLL and Low PLL) for 21 days, in addition to a negative control (no PLL) and a positive control of 17β-estradiol (E2). The P_1 adults exhibited a significant decline in reproductive activity (fecundity over 21 days of exposure) in poultry litter exposed and 17β-estradiol (E2) treated groups compared to the negative control. P_1 males had a significant reduction in the expression of their secondary sex characteristics in all treated groups compared to the negative control, thus likely affecting their breeding behavior and success. Vitellogenin production (an egg yolk pre-cursor protein used as a biomarker to determine estrogenic exposure in male fish) was significant and increased in a dose-dependent manner with respect to the Low, High, and E2 treated male fish. Endogenous steroid hormone levels of E2 increased in males and females, however levels of testosterone (T) increased in females but declined in males.

In the F_1 Generation, both the Low PLL and E2 exposed larval fish from Low PLL and E2 exposed P_1 Generation groups had significantly lower GSI and aromatase activity levels. Gender ratio was skewed toward female in both the E2 exposed groups regardless of whether the P_1 Generation was also exposed to E2. My results demonstrate that exposure to poultry litter leachate may induce endocrine disrupting responses with respect to reproductive capacity in fish populations.

Keywords: endocrine disruptors transgenerational poultry litter fathead minnow

1. Introduction

1.1 Anthropogenic sources of endocrine disrupting compounds

Environmental contaminants are known to induce severe adverse effects in exposed organisms (Segner et al. 2005; Matthiessen 2003; Tyler et al. 1998; Sumpter 1995; Colburn et al. 1993). Over the past 15 years, research on endocrine disrupting compounds (EDCs) has demonstrated that prolonged exposure, or even short-term encounters with potent compounds, can severely impact the health and condition of any organism, aquatic or terrestrial (Ankley et al. 1998; Campbell and Hutchinson 1998; Jobling et al. 1998; Tyler et al. 1998; Sumpter 1995). Along with other anthropogenic sources of EDCs, the intensive development of animal-based agriculture creates waste effluent that feeds into run-off waters. Coupled with the introduction of new and emerging contaminants into our water systems, a slew of compounds have been introduced into the aquatic environment which may impart a variety of insults to various physiological mechanisms of exposed populations. Of particular interest are impacts on reproduction in aquatic species that may span multiple generations, as many EDCs are persistent and toxic in aquatic environments. Yet, few data are available that provide detailed information on populations as a whole (Kidd et al. 2007; Miller and Ankley 2004).

1.2 Laboratory fish full life-cycle tests

Laboratory-based whole-life cycle tests can be labor intensive, time-consuming, and as such are dictated by the life cycle of the organism where short generation times are ideal. However, the information they provide is invaluable to developing strategies and

discovering new tools to aid in assessing and maintaining the health of watersheds. Although the short-term acute response is known for risks associated with EDCs, the long-term chronic impact across generations is not. Since many EDCs target the reproductive axis, tools are needed and data are required on transgenerational responses that will provide information on reproductive capacity, the development of offspring, and the ability of the offspring to grow, develop, and reproduce normally.

Chronic exposure to environmental EDCs such as estrogens and androgens has been shown to induce abnormal reproductive developmental effects in a variety of fish species (Anway et al. 2006; Seki et al. 2005; Zhong et al. 2005; Schwaiger et al. 2002; Zillioux et al. 2001). Several testing guidelines have been established to investigate the impacts of EDCs on subsequent generations of an exposed parental lineage. For example, the fish full life-cycle test (FFLC) has been implemented by the Organization for Economic Cooperation and Development (OECD) and others to establish a standardized testing procedure for laboratories across the globe to assess EDC impacts. This test requires exposure to a contaminant throughout the life cycle of the fish (from egg to adulthood) and includes exposures to their offspring to adulthood (U.S. EPA 2002b). Zhong et al. (2005), Seki et al. (2005) and Länge et al. (2001) utilized this testing procedure with three distinct species and three different EDCs. The results yielded from these exposures of diethylstilbestrol (DES), 17β-estradiol (E2), and 17α-ethinylestradiol (EE2) were that both DES and E2 have significant effects on the reproductive capacity. In addition, DES exposure affects multiple generations with reduced abnormal sexual differentiation, reduced survival, reduced testosterone levels, and elevated E2 levels for both adult and offspring (Seki et al 2005; Zhong et al. 2005). Without implementation of

a standardized testing design, the needed data on key reproductive parameters that are targeted by specific EDCs would be much more difficult to obtain. Life-cycle tests also help in the development of risk assessment models while gaining a better understanding of the impacts these compounds have in the environment.

1.3 Laboratory fish partial life-cycle tests

Other studies have explored similar exposure regimes using a partial life-cycle (PLC) test or transgenerational design. These studies utilize an experimental design that is defined as exposing the organism to a given compound or mixture during the adult stage, followed by exposure to the offspring of these fish through to their adult stage (U.S. EPA 2002a; ASTM 2000; U.S. EPA 1994; OECD 1992). The key concept to using these studies to determine the impacts of EDCs is to encompass the stages of development that are most susceptible to EDC effects. The most sensitive endpoints are utilized such as larval growth and development, sexual differentiation as manifested in gender ratio, adult breeding fitness, and secondary sexual characteristics. Physiological mechanisms known to be under control of the hypothalamic-pituitary gonadal axis and vitellogenin expression in males and juveniles are also used as indicators of estrogenic exposure (Robinson et al. 2004; Foran et al. 2002; Zillioux et al. 2001). Both Robinson (2004) and Brion (2004) implemented partial-life cycle tests and provided valuable data on key estrogenic markers and environmentally relevant concentrations of 17β-estradiol on exposed sand goby (*Pomatoschistus minutus*) and zebrafish (*Danio rerio*) populations. The value of these tests is to provide similar information to that derived by a FFLC test, but to use fish species that are indicators and may have longer life-cycles that are not as easily implemented into FFLC tests.

1.4 Exposure regimes under environmental conditions

For wild populations, exposure to only one EDC during the life-cycle is unlikely. Instead, fish encounter contaminants in aquatic systems from a variety of sources. For example, sources include pulp and paper mill effluent, sewage treatment plant effluent, animal feed lot effluent, or run-off from agricultural fields amended with manure or litter. In essence, wild fish populations may be exposed to a mixture or cocktail of contaminants that may come from a variety of anthropogenic sources that feed into natural water systems. Specific physiological responses to contaminant exposure such as those listed include the reduction of gonadal tissue growth, reduced hormone levels, induction of vitellogenin in males, changes in secondary sexual characteristics, altered behavioral displays, reduced ability of males to compete for mates, reduced fecundity, and skewing of offspring populations toward a majority of females (Martinović et al. 2007; Orlando et al. 2007; Jensen et al. 2006; Liney et al. 2005; Orlando et al. 2004; Soto et al. 2004; Toft et al. 2004; Toft et al. 2003; Orlando et al. 2002; Parrott and Wood 2002). Consistent endocrine disrupting effects are prevalent even though these reports have been from several geographic locations, from a variety of effluent sources, and from an array of fish species. However, the target chemical or chemicals as the causative agent remains unclear. One hypothesis focuses on synthetic or natural estrogens excreted in urine and feces from both human (sewage treatment facilities; Kolpin et al. 2002) and livestock (animal feed-lot effluents, or agricultural run-off; Lee et al. 2007).

In the Chesapeake Bay region, agricultural fields are commonly amended with poultry litter. Previous studies in the laboratory have shown EDC effects in fathead minnow when exposed to no-till poultry litter applied to agricultural fields (Yonkos

2005). In addition, previous laboratory research has also shown evidence of endocrine disruption in larval fish exposed to poultry litter and then grown to adulthood (Chapter 2). These fish exhibited a skewed gender ratio and altered development of secondary sexual characteristics once they reached the adult stage (Chapter 2).

For fish populations with short generation times, EDC effects on individuals can translate to effects at the population level if reproductive function is targeted. Therefore, partial life cycle tests are useful to investigate EDC effects at physiological, behavioral, and population levels. Using this paradigm, the goal of my study were: 1), investigate reproductive performance in the adult fathead minnow exposed to poultry litter leachate as an EDC source, and 2) to determine the overall health and fitness of their offspring when exposed to poultry litter leachate.

2. Materials & Methods

2.1 Experimental design

The experiment was performed during Summer 2007. The experimental design set up was a completely randomized design with 4 treatments for the P_1 Generation, followed by 8 treatments applied to the offspring (F_1 Generation). Aqueous batches of poultry litter leachate at 0.625 g/L (High PLL) and 0.3125 g/L (Low PLL) were prepared daily. Dry poultry litter was weighed to the nearest .001 g, added to 50 L of carbon-filtered tap water and stirred continuously for 4 h. After 4 h of stirring, the leachate was filtered through a fine filter material (0.60 in.; Aquatic Ecosystems, Apopka, FL). The higher, more concentrated leachate treatment was pumped using an 115V/60hz utility pump (Aquatic Ecosystems, Apopka, FL) into each tank within that treatment, and the remaining leachate volume was diluted 1:2 with additional carbon-filtered tap water to

prepare the 1:8 (Low PLL) concentration. Water was added while the High PLL continued to stir. The positive control of 100 ng/L 17β-estradiol (E2) treatment was also prepared daily in a 0.001% ethanol solution. Aliquots of 1 mL of a 100 ng/L solution of E2 were prepared by weighing out 1 mg of 17β-estradiol (Sigma-Aldrich, St. Louis, MO). The E2 was dissolved in 100 mL ethanol, sonicated for 20 minutes and frozen at -20 °C until use. A glass aquarium tank was filled with carbon-filtered tap water to a volume of 95 L and 950 μL of the 100ng/L E2 solution was added, stirred for 20 minutes and then pumped using a 237 gph/22w submersible pump (Aquatic Ecosystems, Apopka, FL) into the appropriate treatment tanks.

All animal procedures were performed under an approved Institutional Animal Care and Use protocol at the University of Maryland, College Park. Prior to initiation of the study, breeding groups consisting of two males and four females were placed into each tank with two pieces of 6.4 mm thick PVC pipe cut in half (see below). If egg production was sustained (spawning occurring every 3 to 4 days, a minimal fecundity of 15 eggs/female/day, >90% fertility; Ankley et al. 2001; Jensen et al. 2001) over the course of the two week acclimation period, the fish would remain for the experiment. If fish did not perform during the course of the acclimation period, they were replaced with new fish and a similar evaluation of reproductive potential would occur (Ankley et al. 2001).

The experiment was initiated after the acclimation period and when two male and 4 female adult fathead minnow (breeding groups, P_1 Generation) were randomly placed into a tank (=experimental unit) with two 6.4 mm thick pieces of PVC pipe cut in half (that measured approximately 123mm X 112 mm X 57 mm) to serve as breeding

substrates. Below each breeding substrate was an 80 mm diameter petri plate (egg tray; Image 3.1) covered in 10 mm mesh to catch any eggs not properly adhered to the substrate (Thorpe et al. 2007; Ankley et al. 2001). Animals were fed *ad libitum* adult zebrafish diet (Ziegler Bros., Inc., Gardners, PA) and frozen brine shrimp slurry. All assessments made on reproductive performance and male secondary sexual characteristics were performed over the course of 21 days.

2.2 Water quality measurements & PLL analysis

Water quality parameters were measured daily using a representative tank for each treatment. The water quality parameters measured were temperature, pH, NO_2, NO_3, and NH_3 using a Hach DR/850 colorimeter (Hach Co., Loveland, CO). The PLL was analyzed for E2 content using an RIA method according to protocols previously described (McMaster et al. 1992). Briefly, prepared samples were run in duplicate then frozen at -20 °C until analysis. Antibody was diluted 1:70 for 48% binding prior to adding to each sample. Samples were then incubated at 4° C with dextran-coated charcoal for 24 hr, then centrifuged at 3,000 rpm. The supernatant was decanted and added to scintillation vials with 5 mL of scintillation cocktail (Perkin-Elmer, Waltham, MA) and counted in a scintillation counter measuring counts per minute (CPM) for five minutes per vial (McMaster et al. 1992). Polyclonal antibodies for estradiol were purchased from Accurate Chemical and Scientific Corporation (Westbury, NY).

2.3 Reproductive capacity

Eggs (F_1 Generation), generated by breeding groups in each tank from the P_1 Generation were placed in treatments dependent upon the treatment of their parent. Table

1.3 describes the specific treatments, but for example, if the parent treatment was a Control treatment, offspring were subsequently placed in a Control treatment and designated ControlControl. Or, offspring were placed in the positive control E2 treatment and the subsequent treatment would be designated ControlE2 where the first name is the parent treatment and the second the offspring treatment.

Eggs attached to the breeding substrates were placed in a 9.5 L tank with the designated treatment and an air stone providing gentle aeration placed directly under the egg mass. The airstone was secured carefully so as to avoid any previous fungal issues encountered in previous studies (see Chapter 1). Eggs that were counted but were found in the egg trays placed below each breeding substrate were also placed in the exposure tank. The exposure for each tank was 21 days. All treatments were prepared in the same manner as was previously described for the P_1 Generation. At the end of the 21 days, all fish were taken off treatment and transferred to a 38 L tank with carbon-filtered tap water, a small stationary filter containing filter media, gentle aeration from an airstone, and were grown up to 6 months of age. All tanks were maintained at 25±2 °C.

2.4 Offspring care and rearing

Larvae were fed artemia until 1 week of age when they were switched to a mixture of artemia and larval diet (250-400 μm; Aquatic Ecosystems, Apopka, FL) for two weeks. For the following three weeks, larvae were fed only larval diet until they were big enough to feed on ground up adult food (Adult Zebrafish diet, Ziegler Bros, Inc., Gardners, PA). Larvae were fed the ground up adult food until approximately 1 month of age when they were switched to non-ground up adult zebrafish diet and remained on this diet until 6 months of age.

2.5 P_1 Generation measures

To assess reproductive success, eggs were counted from the breeding substrates and for each egg tray placed below the substrate for each tank using a handheld counter and inspected visually to determine developmental stage. Fecundity was determined per tank as number of eggs total for each substrate and tray in the tank. Fertility was determined as the number of eggs that reached the eyed-up stage (visual inspection of eggs to note the presence of eyes in the embryonic stage). Percent hatch was determined by the number of larvae generated from fertilized eggs produced.

2.5a Male secondary sec characteristics (P_1 Generation)

Male secondary sex characteristics were measured and recorded on days 0, 7, 14 and 21. Males were lightly anesthetized in 70 mg/L MS-222 and fin clips were made on the pelvic fins to distinguish between males to track individuals each week. Breeding tubercles were counted using a dissecting scope and a handheld counter. Dorsal epithelial pad thickness (as volume in mm^3) was measured with a Fowler Ultra-Cal II digital calipers (Sylvac Inc., Crissier, Switzerland). Weight was taken to the nearest 0.01 g. Total length (snout to tail end) and interocular distance were also measured (day 21 only) using the Fowler Ultra-Cal II digital calipers (Sylvac Inc., Crissier, Switzerland).

2.5b Condition factor (P_1 Generation)

Condition factor was assessed from body weight as weight (g)/length $(mm)^3$ X 100. At sacrifice, gonadal tissue and livers were dissected and weighed to the nearest 0.001 g to assess gonadosomatic index (GSI) and hepatosomatic index (HSI).

2.5c Steroid hormone analyses (P_1 Generation)

To measure steroid hormone levels, at sacrifice, animals were euthanized in buffered MS-222 (100 mg/L with 200 mg $NaHCO_3$/L). Blood was collected from each fish by making a vertical incision at the vent. The blood was drawn into heparinized hematocrit tubes and centrifuged at 10,000 g for 10 min. Plasma was then drawn from the hematocrit tube and stored in microcentrifuge tubes prepared with heparin and aprotinin and stored at -80° C until further analysis. 17β-estradiol (E2) and testosterone (T) were analyzed in plasma of males and females using EIA assay kits purchased from Cayman Chemicals (Ann Arbor, MI) and Assay Designs (Ann Arbor, MI), respectively. Sensitivity, accuracy and precision were also determined for each assay kit. Briefly, samples from each fish were divided into two aliquots, one for each steroid hormone analysis. Volumes were recorded for each and samples were double ether extracted, brought up in assay buffer and diluted accordingly based on validation results. Samples for the E2 assay were diluted 1:100 for females and 1:5 for males. Samples run for testosterone analysis were prepared in a similar manner as for the E2 assay; however, samples were diluted 1:50 for treated males and females. Females in the control group were diluted 1:10 and 1:80 for control males prior to assaying. Radiolabelled E2 and T were prepared in assay buffer for determination of extraction efficiency and were 92% and 80.6 %, respectively.

2.5d Vitellogenin in males (P_1 Generation)

Vitellogenin in male fathead minnow was analyzed using an ELISA for fathead minnow purchased from Biosense Laboratories, Inc. (Bergen, Norway). Samples were run in duplicate at three dilutions, 1:1,000 for control males, 1:20,000 for Low PLL males

and 1:25,000 for E2 and High PLL males. The VTG assay was validated for the fathead minnow prior to analyses using untreated males and females to narrow down the dilution ranges and was repeated to determine interassay variability (Nilsen et al 2004). Sensitivity, accuracy and precision were also determined.

2.6 Offspring measures (F_1 Generation)

Offspring survival was measured after 1 and 2 months from hatch. Individual fish were counted three times for each tank. Survival was calculated as number of larvae at 1 or 2 months out of number of larvae hatched from each tank. Survival was determined for each block of the experiment due to the staggered times at which hatching took place. A block of the experiment is indicated by a separate experiment performed at two distinct time periods, encompassing two 21 day intervals. Block I is the first 21 day exposure and block II is the second 21 day exposure. Survival to at least one month of age in fish generally indicates a higher probability that the organism will survive to adulthood (Houde 1997).

2.6a Condition factor (F_1 Generation)

To measure condition factor and interocular distance for the F_1 Generation, animals were euthanized at 6 months of age with an overdose of buffered MS-222 (100 mg/L and 200 mg/L $NaHCO_3$). Measurements were recorded for length (to the nearest .01 mm) using Fowler Ultra-Cal II digital calipers (Sylvac Inc., Crissier, Switzerland) and weight to the nearest 0.01 g. Condition factor was measured as body weight (g)/ length $(mm)^3$ X 100 (McDonald et al. 2000). The distance between the eyes or interocular

distance, was measured to the nearest 0.01 mm using Fowler Ultra-Cal II digital calipers (Sylvac Inc., Crissier, Switzerland).

2.6b Aromatase activity (F_1 Generation)

To measure aromatase activity, brain tissue was removed and placed into 100 µL of a potassium phosphate buffer (pH 7.4), homogenized for 20 s using a Fisher® Powergen 125 homogenizer and then placed at -80°C until further analysis for aromatase activity. Aromatase activity was analyzed using a method modified from Thompson and Siiteri (1974) and reported per mg of protein. A 3 µL aliquot of homogenate was also set aside and diluted 1:10 and analyzed with a BCA Protein Assay Reagent Kit (Pierce Chemicals, Dallas, TX). Protein samples were prepared on a 96-well microtiter plate and analyzed at 570 nm on a spectrophotometric plate reader. Aromatase activity was calculated and mg of protein was determined to standardize per brain homogenate.

2.6c Gender ratio (F_1 Generation)

To determine gender ratio, at sacrifice, all F_1 fish were visually inspected to verify gender. Immature or ambiguously gendered fish were prepared for histological analysis by sectioning the whole body and removing the head and tail (incisions were made at the anterior end, just beyond the operculum and at the posterior end at the terminus of the gut package/vent). Animals were divided into "small" (28-38 mm length) or "large" (39-50 mm length) and were placed into 10 % buffered formalin. Each fish was prepared histologically with a hematoxylin and eosin stain (American Histo Labs, Gaithersburg, MD). For "small" fish: two, 5µm sections were prepared, with 600 µm between each section. For "large" fish: two, 5 µm sections were prepared with 1000 µm between

sections. Gender ratio was verified histologically on the gender of gonadal cell types (Brion et al. 2004).

2.6d Gonadosomatic index and hepatosomatic index (F_1 Generation)

To measure gonadosomatic index (GSI) and hepatosomatic index (HSI) at sacrifice, if animals were not gender ambiguous, livers and gonadal tissue were removed from 15 representative fish from each replicate per treatment, and weighed to the nearest 0.001 g. GSI was calculated as gonad weight (g)/body weight (g) X 100 and HSI was calculated as liver weight (g)/body weight (g) X 100.

2.6e Male secondary sex characteristics (F_1 Generation)

Male secondary sex characteristics were assessed at sacrifice, for male fish. If they possessed breeding tubercles, they were counted and dorsal epithelial pad volume was measured to the nearest .01 mm^3 using a Fowler Ultra-Cal II digital calipers (Sylvac Inc., Crissier, Switzerland).

2.7 Statistical analyses

All data except gender ratio and P_1 Generation male secondary characteristics were analyzed for differences among treatments using analysis of variance followed by Tukey's test for mean separation. Chi-square analysis was used to analyze gender ratio. For the P_1 Generation, male tubercles and epithelial pad were analyzed over time. Comparisons were made for treatments at each time interval using the sequential Bonferroni test for mean separation. Where data did not meet the requirements for normality (non-normal distribution of data, unequal variances, etc.), the appropriate transformations were made. For the P_1 Generation, log transformations were made for

E2, T and E2/T ratio data. Fertility data were arcsine transformed. For the F_1 Generation, aromatase data were log transformed and survival data were arcsine transformed. Data are presented as back-transformed unless otherwise noted. All ANOVA statistics for the P_1 Generation are presented in Appendix I. All ANOVA statistics for the F_1 Generation are presented in Appendices II. All statistical analyses were performed using SAS® 9.1 (Cary, N.C.) and evaluated at $\alpha \leq 0.05$ level of significance.

3. Results

All water quality parameters fell within the acceptable ranges for water quality parameters including pH, NO_2, NO_3, and NH_3, based on U.S. EPA Quality Criteria for Water (2008). For the P_1 Generation, only three mortalities were recorded. For the F_1 Generation block I, there were no offspring for the HighControl treatment group due to low egg production from the High PLL treated parental breeding groups. Due to difficulties in maintaining consistent temperatures during the early stages of development, one tank, or replicate was lost from the block II HighControl treatment. Steroid hormone analysis of the PLL resulted in a significant concentration of E2 at ~125 µg/kg dry weight. Results were corrected based on a 25% moisture content of the litter leachate.

3.1 P_1 Generation Measures

Fathead minnow exposed to PLL and E2 for 21 days showed a significant reduction in reproductive capacity. Most clearly, cumulative fecundity was reduced in the High PLL, Low PLL, and E2 treatments compared to the Control ($F=2.74$; $df=3,20$; $p<0.0001$; Figure 3.1). Lack of egg production for prolonged periods (> 4 days) was observed in all PLL and E2 treatments. The Control treatment breeding groups

reproduced and had batches of eggs every 3-5 days. As a result of these prolonged periods of no egg production, fecundity per treatment was significantly reduced by 53% in the Low PLL ($p=0.0002$), reduced by 49% in the High PLL ($p=0.0008$), and reduced by 68% in the E2 treatment ($p<0.0001$) compared to the Control. Fertility for all treatments was above 95% and % hatch was above 95% for all treatments except the E2 treatments which was 80% ($F=0.42$; $df=3, 80$; $p=0.0001$; data not shown).

Exposure to High PLL, Low PLL, and E2 for 21 days significantly impacted secondary sex characteristics in males ($F=0.30$; $df=3, 186$; $p=0.04$, Figure 3.2A). Differences were observed between treatments at similar days. Deviations between treatments begin to appear at day 14 where the E2 treatment males had significantly fewer tubercles than Control males. On day 21, differences were observed between the Control treatment males and the High and E2 treatments ($p=0.0008$ and $p=0.0008$, respectively). On day 21, differences were also observed between the Low PLL and the E2 treatment ($p=0.005$). In addition, dorsal epithelial pad volume showed a decline for all males in the Low PLL, High PLL, and E2 treatments ($F=0.30$; $df=3, 176$; Figure 3.2B). Significant differences were observed at day 21 for all treated groups (Low, High, and E2) compared to the Control ($p=0.009$; $p=0.02$; and $p=0.02$, respectively).

Previous studies have used interocular distance (IO) as a sexually dimorphic indicator to determine endocrine disruption in fish species (Orlando et al. 2004). I measured interocular distance in males on day 21. A significantly lower interocular distance was measured for the E2 treatment compared to the Control ($p=0.03$; $F=0.15$; $df=3, 43$; data not shown). No differences among treatments were observed for condition

factor (data not shown; F=3.39; df=3, 134; p=0.20) but the High PLL treated fish had the lowest condition factor compared to all other treatments.

GSI differed significantly between males and females ($p<0.0001$; Table 3.2). For female fathead minnow, differences were observed only between the Low and High PLL treatments where the Low PLL treatment females had a significantly higher GSI value than females in the High PLL treatment group (F=1.51; df=3, 83; p=0.01). GSI did not differ between males in any treatment groups (p=0.70). HSI for male and female fathead minnow differed significantly (p=0.01; Table 3.2). For female fathead minnow HSI, differences were observed between the High PLL and the E2 treatment (p=0.02; F=2.84; df=3, 83). HSI did not differ between treatments for males (p=1.0).

A significant difference in levels of E2 in females compared to males was observed (F=2.32; df=3,83; $p<0.0001$; Table 3.3). In female fathead minnow, differences were observed between the Control and E2 treated females (p=0.01), between the E2 and High PLL females (p=0.03) and between the E2 and Low PLL females (p=0.05; Table 3.3). Male fathead minnow E2 levels were not different among treatments (F=0.19; df=3,38; p=0.99). Extraction efficiency for E2 was 92%. The sensitivity or LOQ of the E2 assay was 14.7 pg/mL and the minimum detection level (MDL) was 2.5 pg/mL. The repeatability precision was 48%.

Differences were observed between male and female fathead minnow for testosterone levels (F=1.85; df=3, 84; $p<0.0001$; Table 3.3). For female fathead minnow, testosterone levels increased with increasing concentrations of poultry litter and the E2 treatment. Differences were observed between the Control and all treated groups ($p<0.0001$). For males, only the High PLL group differed significantly from the Control

group (F=2.41; df=3, 41; p=0.04). Extraction efficiency for testosterone was 81%. The LOQ for the testosterone assay was 15.6 pg/mL and the MDL was 4.4 pg/mL. The interassay variability was 25%.

The E2/T ratio differed significantly between males and females (F=3.21; df=3, 82; p=0.02; Table 3.3). For female fathead minnow for both Low and High PLL treated fish, a significantly lower E2/T ratio was observed compared to Controls (p=0.003 and p=0.02, respectively). No differences in the E2/T ratio were observed among male treatments (F=0.49; df=3, 40; p=0.97).

Vitellogenin expression was measured in all male fathead minnow and the High PLL and E2 treatments resulted in a significant expression of plasma vitellogenin compared to the Controls (p=0.03 and p=0.0002, respectively; F=3.27; df=3, 41; p=0.0004; Figure 3.3). The Control and Low PLL plasma vitellogenin in males was not significantly different. VTG was analyzed using a monoclonal goat anti-rabbit fathead minnow antibody. The sensitivity or LOQ of the assay was 0.08 ng/mL and the MDL was 0.9 ng/mL. Repeatability precision was 16 %.

3.2 F_1 Generation Measures

Survival between experimental blocks and among treatments did not vary significantly (F=0.80; df=6, 29; p=1.0; Table 3.4). Absolute survival in block I was observed to be between 20-41%. Block II survival ranged from 27-43% survival. Percent survival for testing of this nature should be greater than 80% (Ankley et al. 2001). However, survival numbers are lower than to be expected based on densities at hatch and some culls throughout the first and second months of development. Appendix III shows a graph of density of fish compared to survival at two points in time. This graph

demonstrates that survival is not based on density (ie the cause of mortality is not linked to poor water quality as a result of high stocking density). Rather, because numbers of larvae were high at hatch, survival percentages are a reflection of large numbers that were culled to reduce the density or that died naturally to accommodate more manageable numbers per EU early on in development. Additional mortalities after the first two months were on average one fish per month until sacrifice.

Condition factor varied significantly between genders ($p<0.0001$; Table 3.5). The LowLow female fish differed significantly from the ControlControl ($p=0.009$), ControlE2 ($p=0.03$), E2Control ($p=0.004$), and E2E2 ($p=0.0004$) female fish ($F=1.06$, $df=7, 222$). Male fish condition factor differed significantly between the E2E2 group and the ControlControl ($p=0.007$), the ControlE2 ($p=0.02$), the HighHigh ($p=0.03$), the LowControl ($p=0.01$), and the LowLow ($p=0.03$; $F=2.72$; $df=7, 149$).

IO distance was significantly different among genders ($p<0.0001$; Table 3.5). All female fathead minnow had significantly smaller IO distance compared to males regardless of treatment ($p<0.0001$). Significant differences were found among treatments only for females in the ControlControl group compared to the LowLow group ($F=1.24$; $df=7, 221$; $p=0.02$). Male fathead minnow did not differ significantly ($p=0.99$; $F=1.39$; $df=7, 149$).

Aromatase activity in PLL exposed offspring of PLL exposed parents was sexually dimorphic for most treated groups where females exhibited a higher aromatase activity than males but not significantly ($F=3.75$; $df=7,226$; $p=0.70$; Table 3.5). Among females, a significantly different aromatase activity was observed for the ControlE2 compared to the LowLow treatment ($p=0.02$), the E2Control compared to the E2E2

(p=0.05), between the E2E2 and LowLow (p=0.03) and between the ControlE2 and E2Contol (p=0.05). Treatments with overall lowest aromatase activity were ControlE2, E2E2, LowControl, HighControl and HighHigh. No differences among males were observed (F=1.86, df=7, 147; p=0.99).

Gender ratio was skewed toward female in both the ControlE2 and E2E2 treatments with 88 % female compared to 11% male in the ControlE2 treatment (p=0.001) and 84% female and 16% male in the E2E2 treatment (p=0.009; Figure 3.4). All fish sampled for histological analysis had normal development of gonadal tissue (see Images 3.2-3.12). However, one fish from the LowControl treatment group was intersex and had both testicular and ovarian tissue (Image 3.13).

GSI for the F_1 Generation of offspring exposed to PLL was significantly different between gender (F= 2.28; df=7, 90.2; p<0.0001; Table 3.6) where males had a significantly lower GSI across all treatments compared to females. However, only differences among females across treatments were observed with respect to the ControlE2 and LowControl treatments (p=0.02) and the E2Control and E2E2 treatments (p=0.04). For the HSI analyses, no differences were observed with respect to gender due to the large variability among all treatments (F=0.79; df=7, 93; p=0.80; Table 3.6). However, differences among females were observed between the ControlControl and ControlE2 treatments (p=0.02).

The male secondary sex characteristics quantified were the epithelial pad volume (mm^3) and breeding tubercle number. Only males that had these traits visible were included in the analysis. Males in the HighHigh treatment group had significantly larger epithelial pad volumes as compared to epithelial pad volumes for males in the

ControlControl (p=0.005), LowLow (p=0.006) and LowControl (F=2.98; df=7, 89; p=0.01; Table 3.7). Breeding tubercle number for the F_1 Generation males differed significantly between the E2E2 group and the ControlControl (p=0.02), LowLow (p=0.05) and LowControl treatments (p=0.03; F=0.86; df=7, 89; Table 3.7).

4. Discussion

Anthropogenic EDCs originate from a variety of sources. Effluent or mixtures of potential EDCs are of particular concern because the individual components are unknown and can vary due to a variety of factors on a daily basis, and because aquatic organisms are constantly inundated by exposure. In the Chesapeake Bay region, run-off from agricultural fields with poultry litter applied as fertilizer has been shown to contain a significant amount of steroid hormones, 17β-estradiol and testosterone (unpublished data) and that in the laboratory, exposure to poultry litter leachate can severely impact the development of male secondary sex characteristics necessary for successful reproduction (Chapter 2). Poultry litter exposure in a laboratory setting has been shown to induce VTG expression in male fish (Fisher et al. 2003); an indication that the poultry litter mixture contains estrogenic compounds to which these males have been exposed (Yonkos 2005). As such, the EDC components of poultry litter have the potential to cause adverse effects on not only the reproductive biology of adult fish, but subsequently impact the development of offspring from exposed parental generations. For these reasons, I utilized a transgenerational exposure regime under laboratory conditions to assess EDC effects at different developmental stages using poultry litter as an EDC source. I proposed that exposure to an EDC mixture potentially containing steroid hormones would alter

reproductive performance in adult fish. The response of adult reproductive performance to EDCs may then carry through to the offspring generation.

In the P_1 Generation, a significant reduction in fecundity was observed for all treated groups compared to controls. As a consequence, the lack of sufficient egg production production from the P_1 Generation High PLL treatments prevented the comparison of the HighControl treated offspring to other offspring groups. The use of egg trays to catch any fallen or possibly improperly adhered eggs was implemented to maximize fecundity estimates and minimize any bias for this endpoint among treatments (Thorpe et al. 2007). Fallen eggs were counted and set to hatch for the F_1 Generation if viable when counted.

Male fish in all treated groups were observed to be engaging in reproductive behaviors; however, this varied from day to day, and it is unclear within the reproductive repertoire was because of lack of successful egg production or reproductive capacity was compromised. Similarly, Majewski et al. (2002) and Martinović et al. (2007) observed male fish exposed to the EDC, 17α-ethinylestradiol or sewage treatment effluent, engage in reproductive behaviors but at a much more reduced rate than their non-exposed counterparts.

In my study, fertility and % hatch were observably robust for all treated groups with greater than 80% success. This would indicate that if productive spawns occurred within PLL treated water, the male was able to fertilize and protect the eggs successfully even if there were fewer occasions where the male did so in the poultry litter and E2 treated groups.

The presence of male secondary sex characteristics contributes to breeding success in the fathead minnow (Ankley et al. 2001; Länge et al. 2001). With exposure to EDCs, males are vulnerable to the lack of development of secondary sex characteristics (Ankley et al. 2003; Länge et al. 2001; Harries et al. 2000). Results from the P_1 Generation showed not only a difference in tubercle number among treated groups, but a divergence in the number of tubercles that began to appear at day 14 of exposure. Because I evaluated tubercle number at regular intervals over the course of the study, I was able to determine that tubercle number not only differed among treated groups but that since all males initially had a similar number of tubercles, males in the control group gained tubercles and males in the treated and E2 groups lost tubercles with exposure time.

Similarly, with the development of the dorsal epithelial pad, pad dimensions were evaluated at regular weekly intervals during the course of the study. An observed decline in pad volume occurred at day 21. All epithelial pad volumes were measured prior to exposure and pad volumes were similar among all males. However, as the experiment progressed, all PLL and E2 treated males lost volume of the dorsal epithelial pad. The combined loss or lack of both of these male characters after 21 days of exposure to the PLL indicates that these exposed males may be at a disadvantage when competing for mates and nesting territories.

To my knowledge, this is the first study to present the evaluation of these traits, by utilizing measurements at regular intervals to note changes over time. In addition, this is the first experiment to that implicates the precise measurements of the pad volume to determine effects over time rather than just the presence or absence of this trait and as an

indication of endocrine disruption. Smith and Murphy (1974) observed changes in dorsal epithelial pad tissue to determine the origin of the function of this morphological trait. Utilizing the methodology of Smith and Murphy (1974), I challenge the conventional idea of measuring dorsal epithelial presence as a function of male breeding success. The simple presence of a pad may not be enough to evaluate the effectiveness of the male fathead minnow ability to maintain a nest; the length, height, and width are as important if not more due to the fact that a male fathead minnow utilizes this morphological character in three dimensions to be most effective.

Previous studies provide evidence that endocrine disruption affects the IO distance as a sexually dimorphic character (Orlando et al. 2007; Orlando et al. 2004). Significant differences for male IO distance in the P_1 Generation were observed only between the control and E2 exposed males. For the P_1 Generation IO distance was measured in males only to determine endocrine disruption as it is normally sexually dimorphic (Orlando et al. 2004). Thus, IO distance was a reliable measure of endocrine disruption in males and may provide an additional, robust morphological indicator (aside from the VTG response) to determine endocrine disruption.

Gonadosomatic index and hepatosomatic index provide structural and morphological indicators for gonad and liver health in fish (McDonald et al. 2000). These morphometrics are general body measurements to assess weight and health by comparing organ weights to body weight. HSI can be used as a biomarker in endocrine disrupting studies by providing comprehensive information on liver tissue response to contaminant exposure. The liver is the main organ where exogenous compounds are metabolized. GSI measurements afford a direct assessment of gonadal tissue development. Due to the

dramatic variation in gonad size that occurs among female fish and the disparity in gonadal tissue size between genders, GSI was evaluated separately for males and females.

Female GSI values differed significantly only between the Low and High PLL treatments. This observation may be due to the large variability among individual development of ovarian tissue, which in turn can be related to varying levels of hormones that continue to fluctuate during the reproductive cycle (Peter and Yu 1997). Several studies also demonstrate that steroid hormones can vary up to 30-fold from individuals at the same site during gonadal recrudescence (Folmar et al. 1996). For males, because testicular tissue develops in a more consistent fashion, and observed hormone levels do not fluctuate as dramatically, a more monotonic response is possible with male GSI values.

Metabolic activities and energy storage are inherent to liver function and growth (Dethloff and Schmitt 2000). Although alterations of liver size may be due to environmental stressors, additional factors may impact HSI values such as nutritional stores, metabolic rates, and storage capacity (Daniels and Robinson 1986). Differences in HSI values in females may also be attributed to the fluctuation in hormones observed throughout the reproductive cycle: HSI values change significantly due to the liver's role in vitellogenesis (Scott and Pankhurst 1992).

Because circulating steroid hormone levels are driven by the hypothalamic-pituitary-gonadal axis (HPG) in fish, reproductive mechanisms, which include sexual differentiation, gamete development at adulthood, formation of secondary sexual characteristics which aid in spawning behaviors, are strongly reliant upon proper levels of

these hormones (Young et al. 2005). In my study, 17β-estradiol levels in adult female fathead minnow in the control and poultry litter exposed treatments exhibited no change whereas E2 levels were highest in E2 treated females. These results suggest that levels of estrogenic contaminants possibly within the poultry litter leachate are not at sufficient levels to alter the synthesis of this hormone. In contrast, the E2 positive control females that exhibited an increase in E2 levels may indicate that the concentration of 100 ng/L is higher than what may be present in the litter and has caused the considerable increase in E2 as compared to PLL and negative control treated females.

Previous studies have shown with exposure to estrogenic EDCs or effluent, circulating levels of E2 decline in both females and males (Douxfils et al. 2007; Martinović et al. 2007). However, exposure to cadmium, which targets the ovaries and acts as a reproductive toxin, caused circulating E2 levels to increase in females exposed to 12.5 µg/L (Sellin and Kolok 2006). Levels of E2 in female fathead minnow exposed to cattle feed-lot effluent or a stream receiving agricultural run-off also increased compared to controls (Orlando et al. 2004). In the latter cases, where E2 levels show an increase compared to control females, levels of estrogenic compounds in the exposure mixtures may be higher than threshold levels present endogenously circulating in female plasma. This high E2 level exposure may cause the feed-back mechanisms along the HPG axis to up-regulate receptors and to increase signaling hormones (GnRH, LH) so as to meet the demands of the increase in circulating levels of hormone entering the blood from the external environment.

In females, a significant increase in T levels also coincided with an increase in PLL concentrations and E2 in a dose-dependent manner. Positive feedback on the HPG

axis would warrant this trend as levels of T are lower in females. T levels in male fathead minnow however, were significantly different in the Low PLL compared to controls. Orlando et al. (2004) observed a similar trend in male fathead minnow exposed to cattle feed-lot effluent and a stream receiving agricultural run-off. The highest levels of T were expressed in males at the stream site and significantly lower T levels were expressed by males at the run-off site.

Previous studies have employed the E2/T ratio to measure the extent of endocrine disruption. In turn, these measurements may relate well to observations in GSI values, aromatase activity, or secondary sex characteristics (Orlando et al. 2004; Harries et al. 2000; Nichols et al. 1999; Folmar et al. 1996). My results show female fathead minnow E2/T ratios were significantly reduced for Low PLL exposed fish. This may be due to the reduction in E2 and elevation of T in these female fish. Female fathead minnow exposed to cattle feed-lot effluent or a stream receiving agricultural run-off also had a defeminized sex hormone ratio due to reductions in E2 synthesis by these females (Orlando et al. 2004). No significant differences were observed in male fish E2/T ratios which may be due in part to the lack of significance in synthesis of E2 or T despite elevations of E2 and declines in T overall for exposed males.

Further evidence to describe the estrogenic activity of the PLL is the expression of VTG in male fathead minnow. VTG has been used extensively in endocrine disruptor research as a biomarker of exposure to estrogenic compounds (Fenske et al. 2005; Seki et al. 2005; Nash et al. 2004; Seki et al. 2003; van den Belt et al. 2002; Sohoni et al. 2001; Tyler et al. 1999; Harries et al. 1997; Harries et al. 1996; Sumpter 1995; Purdom et al. 1994). VTG is a reliable, robust, and easily quantifiable method to determine estrogen

exposure (Tyler et al. 1999). The legacy of the use of VTG, further strengths, and discussions of the importance of the development of sensitive assays to measure VTG are discussed elsewhere (Chapter 1; Chapter 2). A significant expression of vitellogenin was observed in male fathead minnow exposed to the High PLL and E2 treatments compared to Controls. Levels of VTG expressed in Low and High PLL exposed males were on the order of what has been observed for circulating vitellogenin in normal female fathead minnow (15 mg/mL; Jensen et al. 2001). 17β-estradiol exposed males had circulating VTG levels approximately twice that of a normal circulating female. The expression of VTG in males has become so heavily utilized further strengthens the reliability of this biomarker for aquatic toxicology studies.

The staggered experimental design dictated the hatching of offspring into two separate blocks causing a portion of offspring to be older by at least 1 month from others. As such, survival of offspring was measured at one interval but covered the range of one month or two. Survival varied among all treatments but was low (< 50%) for both blocks for the ControlControl treatment. This can be attributed to two possible causes. The first being the large numbers of eggs produced by the parent groups and the rearing chambers (9.5 L tanks and then to 38 L tanks) not accommodating for such large numbers of offspring (~120-250 eggs per replicate). Other treated groups had higher survival percentages in the first block versus the second block (LowLow and HighHigh treatments). Stocking densities at the rearing stage are incredibly important and may largely dictate survival of larvae due to the resources required at this sensitive stage of development (Baskerville-Bridges and Kling 1999). The second reason may be that these numbers were indeed very rough estimates as fish were viewed only by the naked eye

and counted three times to ensure accuracy. Within a larger population of fish, resources are scarcer and growth can be altered by the competitive actions of each individual (Baskerville-Bridges and King 1999). The stocking densities in my study were maintained at high numbers initially to sort out the fittest individuals per treatment. The greatest amount of mortaility naturally occurs during the early life stage of development of fishes and results in selecting out individuals that may not survive otherwise (Houde 1997). It is also well established that body size and temperature are the two variables that have most clearly demonstrated to be related to survival and productivity during early development (Houde 1997). This theory also translates well to microcosm studies as high stocking density should not interfere with normal growth patterns. Shaw et al (1995) states that as high numbers of larval fathead minnow in microcosms were larger in number and resulted in smaller fish size, this large biomass did not alter development and therefore growth was found to be not related to density dependent effects. In addition, compensatory growth occurs in larvae that start out as smaller individuals once they get past the initial selective phase (Springate and Fromage 1975).

Within the HighHigh and HighControl groups, fewer males hatched compared to females and although not significant, this can lead to increased growth among males driving competition among them. This has been observed with respect to larval fish exposed to PLL (Chapter 2). For males in the E2E2 and ControlE2 groups, a significant difference in gender ratio was observed (this will be discussed further) and this may be related to the impact of the E2 treatment on growth of these males. Also related to growth, IO distance in the F_1 Generation was measured in both male and female fish to determine not only the impacts from treatment exposure but to verify the sexual

dimorphic nature of this trait. A significant difference observed between genders indicated the sexual dimorphic nature of this morphological trait.

The synthesis of sex steroids via activity of the aromatase enzyme is a critical factor for regulation of many physiological functions in fish including sexual differentiation, development, and reproduction (Halm et al. 2002). For most of the F_1 Generation treated groups, a normal, sexually dimorphic expression of aromatase activity was observed where females had higher aromatase activity compared to males. However, aromatase activity in female F_1 fish was severely reduced in the E2 treated groups (ControlE2 and E2) compared to controls. LowLow and HighControl female fish had statistically significant higher aromatase levels than all other groups except the ControlControl. Bream (*Abramis brama*) sampled from a stretch of the Elbe river characterized by elevated concentrations of metals and organic contaminants, had a similar response where aromatase activity was diminished in female fish and correlated with lower GSI values (Hecker et al. 2007). The lack of response in male fish is also similar to results found by Hecker et al. (2007) in that males exhibited no significant difference for aromatase activity. Interestingly, both Hecker et al. (2007) and Noaksson et al. (2003) found a positive correlation between a decline in aromatase activity, decrease in GSI, and reduced steroid hormone levels for female fish exposed to the contaminated Elbe River or a refuse dump in Sweden, respectively.

My results also show a similar trend where GSI values are severely reduced in female fish exposed to E2 (E2E2 and ControlE2) and Low PLL compared to their control counterpart treated groups (see section discussing GSI and HSI F_1 Generation). This could indicate that these female fish are sensitive to exposure at a previous

developmental stage and that at adulthood, these females are now at a significant disadvantage for becoming sexually mature. Lowered aromatase activity and lower GSI suggests a compromised ability for E2 synthesis and subsequently a deficiency in ovarian maturation.

Gender ratio analysis provides insight into the population dynamics of the offspring generation, but also offers the opportunity for coupling the parental generation exposure effects with offspring generation response. Gender ratio was significantly skewed toward female in the E2 treated groups (E2E2 and ControlE2); however, in the LowLow treated group, the proportion of males to females is lower than other poultry litter groups.

The period of sexual differentiation for fathead minnow is approximately 0-20 days post-hatch (van Aerle et al. 2002). As previously described, within this time period the gonadal tissue is susceptible to the effects of exogenous steroid exposure (particularly with E2) where male presumptive gonads can develop female germ cells (Zhong et al. 2005; Brion et al. 2004; Zillioux et al. 2001), female ovarian cavity structure and morphology (Liney et al. 2005), or female secondary sex characteristics and lack of male traits (Chapter 1; Seki et al. 2005; Nash et al. 2004; Parrot and Wood 2002).

In my study, there were several fish observed to be gender ambiguous phenotypically. Gender for these fish was instead verified histologically. Although it was found in only one individual from the LowControl treated group, a presumptive male had developed ovo-testes (Image 3.13). While this population was relatively small (~418 animals), and only one individual among them has developed ovo-testes, it is not clear to

what extent this occurs in natural populations. Furthermore, research is needed to determine if this occurs only when the parental generation is exposed to poultry litter.

Measurement of the GSI values for offspring was assessed in a small subset of fish from each treatment group. Males and females were significantly different where males had a much lower GSI value than females as was expected. The most interesting response, however, was that for the Low and E2 exposed groups. Females in the LowLow group compared to their control counterpart (LowControl) had a much lower GSI value, and similarly for the E2E2 group, GSI was much lower than for the E2Control group. For the control groups, although not significant, a similar trend is observed where the ControlE2 female fish had a much lower GSI than the ControlControl counterpart. Coupled with a lower aromatase activity observed for these treated groups, the combination of compromised GSI and aromatase activity could indicate a reduction in reproductive capacity if these fish were to enter the spawning population.

The F_1 Generation treatment group where males had the most tubercles was the E2E2 group. This may be attributable to the fact that there were so few males in this population (N=6). This trend has been previously observed (Chapter 2) where fewer males in the population generate less of a hierarchical structure among males. Within a population of fathead minnow, once males reach sexually maturity, they begin to seek out territories and their secondary sex characteristics become more developed as they engage in cleaning and nesting behaviors (McMillan and Smith 1974). Most populations of fathead minnow consist of only a few mature males who are able to defend these territories as they out compete weaker more submissive males (Danylchuk and Tonn 2001). In a population with few males, there are more potential territories than males, and

the opportunity to develop these secondary sex characteristics is presented allowing for all males in the population to develop these traits. Interestingly, I did not observe a similar trend for breeding tubercles as was observed for the development of the dorsal epithelial pad. The HighHigh group had one of the larger populations of males for these groups (N=18) so competition among males was potentially less in this treated group.

Conclusions

On the Eastern Shore of Maryland's Chesapeake Bay, there are approximately 1,900 poultry growers and 5,100 poultry houses (Delmarva Poultry Industry 2008). In 2007, Maryland produced 294,800,000 broilers and ranked 8th among the states in the number of broilers produced (Delmarva Poultry Industry 2008). While production figures indicate this multi-million dollar business provides for the economy of the state, removal and/or use of the large amounts of poultry litter continuously generated must be done so in a responsible manner. The persistent nature of steroid hormones determined to be in the litter is an additional concern that must be considered when application of litter to agricultural fields occurs in preparation for the growing season. Colucci et al. (2001b) determined the half-life of E2 in soils to be between 0.2 and 7 days. Lee et al. (2003) observed similar rates in soil-water slurries where $t_{1/2}$ were 0.8-9.7 days for E2. Testosterone also has a half-life within a reasonable time frame to be degraded prior to application of litter (0.3-7.3 days or 2-14 days; Lee et al. 2003 and Colucci et al. 2001a, respectively).

Hakk et al. (2005) suggests composting by amending litter with hay, leaves, and straw to increase microbial colonization and increase aeration. Reported hormone levels were still detectable in extracts 4 to 6 months after composting so although this method is

promising, further research must be done to assess this strategy and optimize this method to be put into best management practices. My results show that E2 is detectable in the poultry litter leachate using RIA methods previously described. While cross-reactivities for the anti-body used are low (<0.07% for E2 pre-cursors and range from 0.12-1.4% with other estrogens, namely E1), and accuracy and precision of the method has been previously tested, limitations for this method and others of this nature exist. Difficulties arise regarding clean-up methods and the ability to measure with confidence, individual components of a matrix such as poultry litter leachate.

My results do show that poultry litter exposure of fathead minnow to poultry litter severely impacts the reproductive performance of adults as well as causing adverse impacts in their offspring throughout the entire life-cycle. Male fish were feminized and in addition, reproductive capacity of females was significantly reduced, where gametogenic output declined steadily over the course of the exposure period. Although the fathead minnow is a fractional spawner, two weeks in the spawning cycle of a fathead minnow is a large portion of the time frame when these animals are reproductive; another indication that adult fish were at a severe disadvantage when exposed to the potential endocrine disrupting compounds from poultry litter.

When EDC impacts carry through to the offspring generation, this may lead to population level effects. My results indicate that the F_1 Generation experienced compromised development and reproductive growth as GSI levels in female PLL exposed fish also had lower aromatase activity. This can lead to an overall reduced reproductive performance. Further, the presence of ovo-testes in one of the fish exposed to the PLL is of concern. Although no direct observations were made to indicate there were carry-over

effects from parental exposure compromising the offspring development, this transgenerational design is an extremely powerful tool that helps provide information to discern these types of effects if they occur. Thus, my study demonstrates that prolonged (across generations) exposure to poultry litter leachate clearly compromises the ability of reproductively mature animals to reproduce. If these effects translate to wild populations, the health of aquatic ecosystems in the Chesapeake Bay region depends upon responsible management of agricultural practices to reduce run-off.

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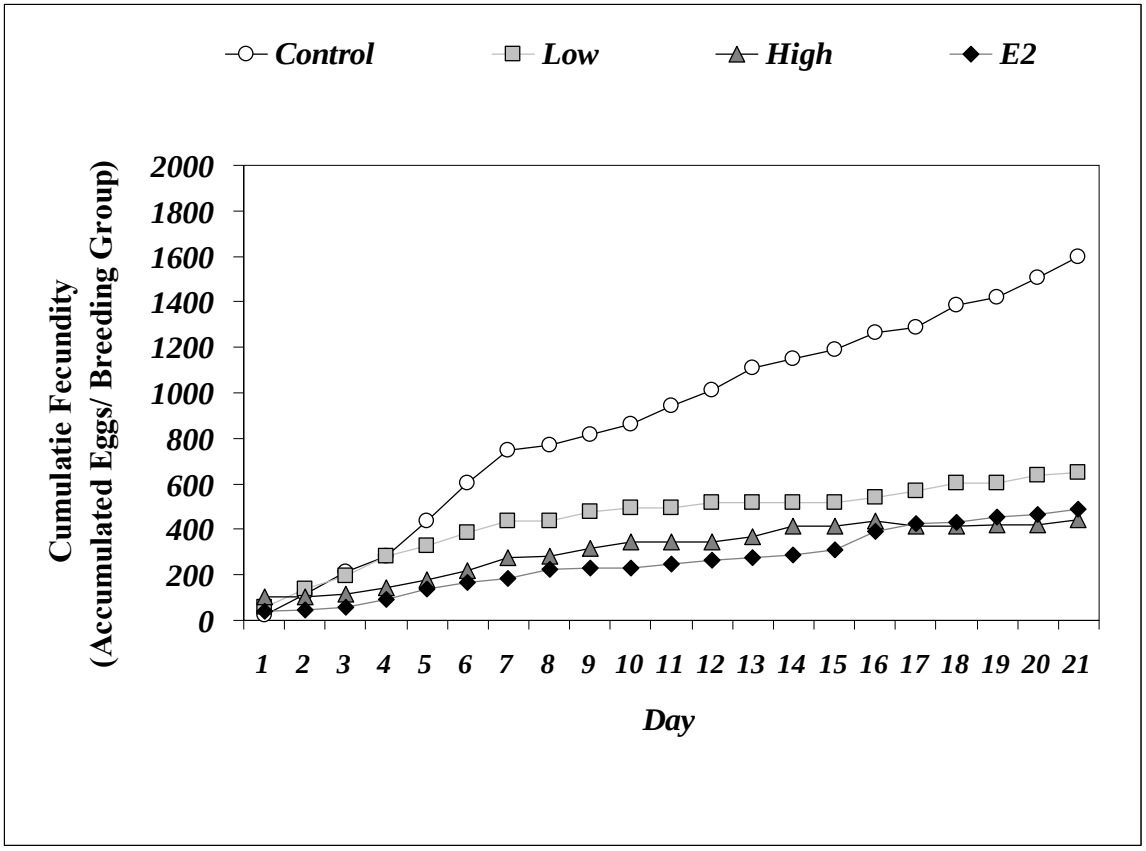
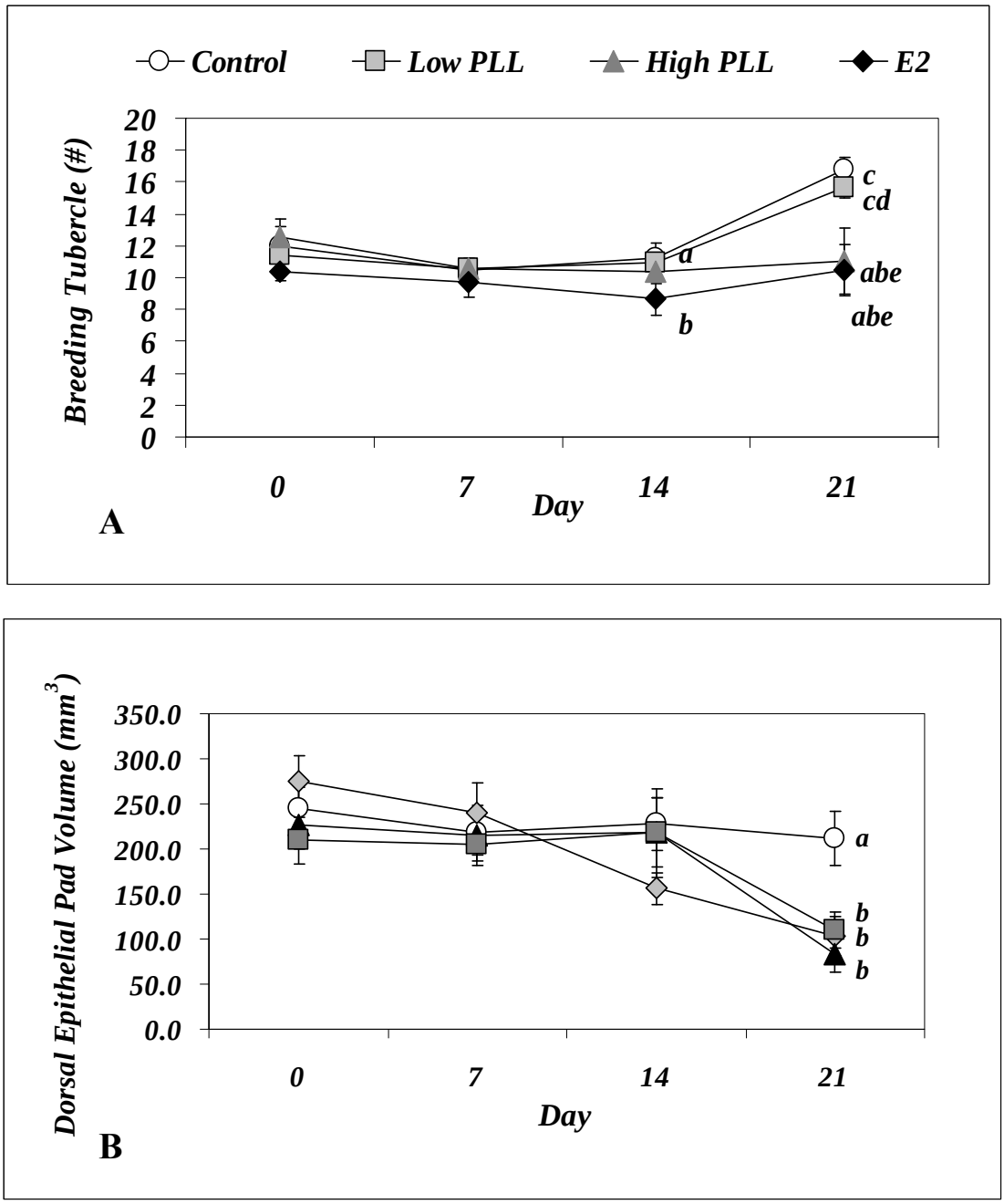


Figure 3.1. P_1 Generation. Cumulative fecundity of the P_1 Generation exposed to 4 treatments: Control, no poultry litter leachate (PLL); Low, (0.3125 g/L PLL; High, 0.6250 g/L PLL; E2. positive 17β-estradiol control. Values are means of 6 breeding groups per treatment.



Figures 3.2A and 3.2B. Breeding tubercles and dorsal epithelial pad volumes of male P_1 Generation fathead minnow exposed to 4 treatments: Control, no poultry liter leachate (PLL); Low, 0.3125 g/L PLL; High, 0.6250 g/L PLL; E2, positive 17β-estradiol control. Values are means of 12 males per treatment. Significant differences are indicated by different letters for each week.

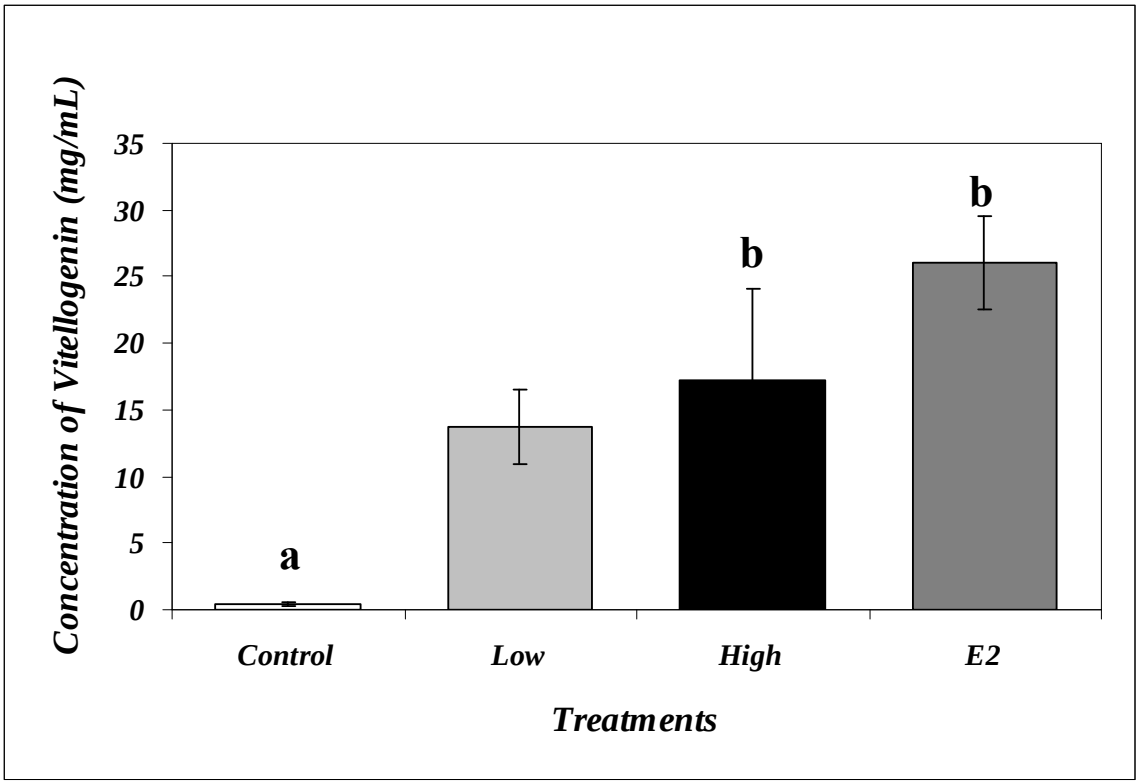


Figure 3.3. P_1 Generation. Vitellogenin expression for male fathead minnow exposed to PLL and E2 for 21 days. Differences between treatments are indicated by different letters. Values are means ± SEM (N=12 males/treatment).

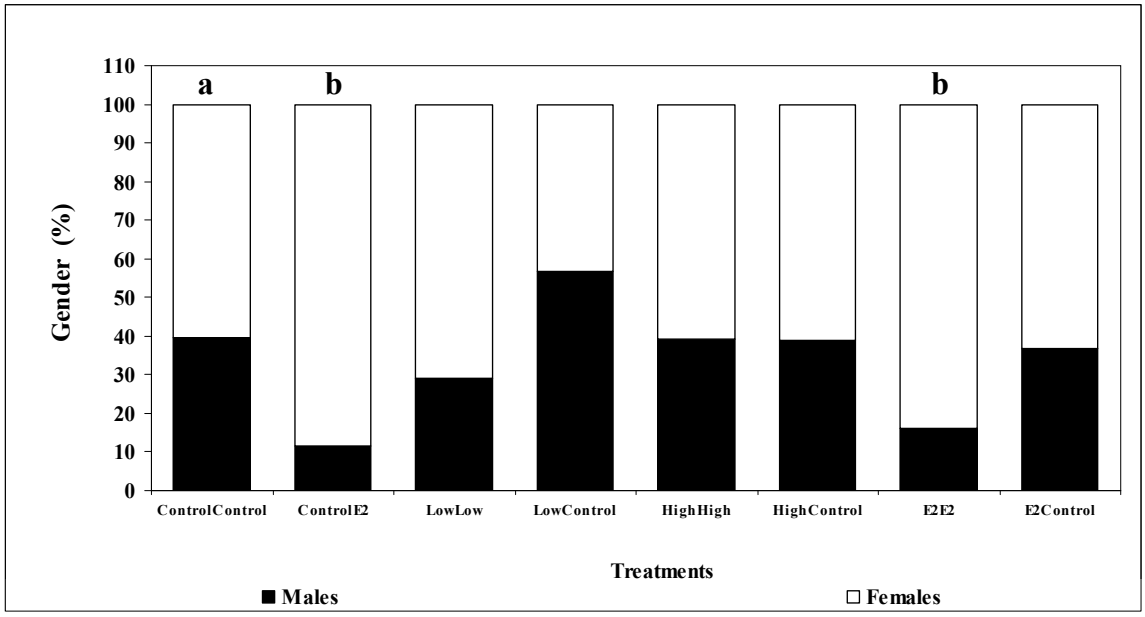


Figure 3.4. F_1 Generation. Gender ratio. Values are percentage of females compared to males in each treatment group. N=11-37 females / treatment and N=8-28 males / treatment. Differences were observed with respect to a skewed gender ratio toward female in the ControlE2 group compared to ControlControl (p=0.001) and between the ControlControl and E2E2 group (p=0.01).

Table 3.1. Treatment designations for F_1 Generation. For each parent or P_1 treatment, offspring that were generated would be placed in either of two treatments. For each of those treatments, three replicates would be generated yielding a total of 24 tanks of offspring.

Option		Parent Treatment		Offspring Treatment Options		Resulting Offspring Treatment Designation
1	If	Control	and	Control	Then	ControlControl
2	If	Control	and	E2	Then	ControlE2
1	If	Low PLL	and	Control	Then	LowControl
2	If	Low PLL	and	Low PLL	Then	LowLow
1	If	High PLL	and	Control	Then	HighControl
2	If	High PLL	and	High	Then	HighHigh
1	If	E2	and	Control	Then	E2Control
2	If	E2	and	E2	Then	E2E2

Table 3.2. Gonadosomatic index (GSI) and hepatosomatic index (HSI) for females and males of the P_1 Generation exposed to 4 treatments: Control, no poultry litter leachate (PLL); Low, 0.3125 g/L PLL; High, 0.6250 g/L PLL; E2, positive 17β-estradiol control. Values for GSI for females are means of N=24 per treatment and males are means of N=12 per treatment. Values for HSI for females are means of N=24 per treatment. Values for HSI for males are means of N=12 per treatment. Significant differences are indicated by different letters.

Parameter	Gender	Treatment			
		Control	Low	High	E2
GSI	Female	12.7 ± 2.39^{ab}	18.3 ± 5.24^{a}	9.08 ± 0.943^{b}	13.4 ± 0.939^{ab}
	Male	0.771±0.146	0.701±0.096	0.697±0.048	0.514±0.055
HSI	Female	1.57 ± 0.196^{a}	2.68 ± 0.653^{ab}	2.54 ± 0.542^{b}	1.71 ± 0.199^{a}
	Male	1.39 ±0.459	1.44±0.228	1.71±0.181	1.25±0.197

Table 3.3 17β-Estradiol (E2), testosterone (T), and E2/T ratio for females and males of the P_1 Generation exposed to 4 treatments: Control, no poultry litter leachate (PLL); Low, 0.3125 g/L PLL; High, 0.6250 g/L PLL; E2, positive 17β-estradiol control. Values for E2, T, and E2/T in females are back-transformed estimates of 24 observations per treatment and males are back-transformed estimates of 12 observations per treatment. Significant differences are indicated by different letters.

Parameter	Gender	Treatment			
		Control	Low	High	E2
17β-Estradiol (E2; pg/mL)	Female	11.59[a]	13.28[a]	12.09[a]	29.69[b]
	Male	2.942	3.552	3.862	4.788
Testosterone (T; pg/mL)	Female	129.52[a]	1876.0[b]	2277.4[b]	4369.9[b]
	Male	11675.3[a]	2029.32[b]	6078.44	3509.91
E2/T	Female	1.93[a]	1.03[b]	0.921[b]	1.28
	Male	1.16	1.72	1.48	2.31

Table 3.4. Density for F_1 Generation fish in block I and block II. Values are numbers at hatch, then numbers at a given interval (1 month or 2 months, depending on block) and density is determined by the percent survivorship or number of fish in each tank after 1 (block II) or 2 (block I) months from hatch.

	Block	Control Control	Control E2	Low Low	Low Control	High High	High Control	E2 Control	E2E2
Mean # at hatch Mean # at month 2 Density (% survival)	I	201 45 29	193 33 28	166 21 20	68 19 33	170 27 23	-	100 38 41	102 23 26
Mean # at hatch Mean # at month 1 Density (% survival)	II	330 153 41	154 64 40	148 39 32	148 27 27	157 23 31	97 26 36	254 56 43	82 38 30

Table 3.5. F_1 Generation condition factor, interocular distance, and aromatase activity. Values are means ± SEM of 12-45 females and 8-30 males per treatment. Significant differences for condition factor were observed between gender and among different treated female groups. IO distance was different between genders and significant differences were observed between treatments for males. Differences for aromatase activity were observed only between female treated groups. Differences are indicated by different letters.

Parameter	Sex	Treatment							
		Control Control	Control E2	Low Low	Low Control	High High	High Control	E2 Control	E2 E2
Condition Factor	Female	0.0009[a]	0.0009[a]	0.0010[b]	0.0010	0.0010	0.0011	0.0010[a]	0.0009
	Male	0.0010[b]	0.0009[b]	0.0010[b]	0.0010[b]	0.0011[b]	0.0013	0.0010	0.0013[a]
IO Distance (mm)	Female	5.46±0.080[a]	5.12±0.080	5.49±0.10 0[b]	5.46±0.12 0	5.53±0.13 0	5.81±0.16 0	5.30± 0.140	5.17 ±0.100
	Male	6.66 ±0.150	5.65±0.190	6.43±0.25 0	6.12 ±0.180	7.23±0.29 0	7.04±0.27 0	6.71 ±0.240	6.42 ±0.350
Aromatase Activity (fmol/hr/mg protein)	Female	603.8 ±75.50	237.0 ±33.17[a]	563.1 ±73.31[b]	350.4 ±58.71	338.5 ±55.42	355.6 ±66.19	560.6 ±62.23[b]	2423.0 ±34.95[ac]
	Male	501.5 ±127.1	229.4 ±47.44	500.3 ±86.98	262.6 ±34.59	429.5 ±55.25	511.7 ±26.18	373.8 ±63.31	374.7 ±97.90

Table 3.6. Gonadosomatic index (GSI) and hepatosomatic index (HSI) for females and males of the F_1 Generation exposed to 8 treatments: ControlControl, ControlE2, LowLow, LowControl, HighHigh, HighControl, E2Control, E2E2. Values are means ± SEM. N=3-11 females per treatment and N=1-8 males per treatment. Differences are indicated by different letters.

Parameter	Gender	Treatment							
		Control Control	Control E2	Low Low	Low Control	High High	High Control	E2 Control	E2 E2
GSI	Female	5.28 ±1.42	2.48 ±1.05[a]	4.90 ±1.44[ac]	8.98 ±0.857[b]	3.20 ±1.25	2.10 ±0.977[a]	8.18 ±2.73[b]	2.34 ±0.758[a]
	Male	0.444 ±0.112	-	0.381 ±0.060	0.546 ±0.135	0.594 ±0.075	0.683 ±0.087	0.647 ±0.070	0.542 ±0.045
HSI	Female	2.52 ±1.20[a]	0.452± 0.081[b]	0.703 ±0.106	1.10 ±0.482	0.604 ±0.164	0.733 ±0.126	0.955 ±0.191	0.784 ±0.129
	Male	0.956 ±0.299	-	0.937 ±0.242	1.08 ±0.167	1.06 ±0.339	0.786 ±0.108	1.15 ±0.200	0.645 ±0.353

Table 3.7. Male secondary sex characteristics for males of the F_1 Generation exposed to 8 treatments: ControlControl, ControlE2, LowLow, LowControl, HighHigh, HighControl, E2Control, and E2E2.

Values are means ± SEM of 5-23 males per treatment. Significant differences are indicated by different letters.

Parameter	Treatment							
	Control Control	Control E2	Low Low	Low Control	High High	High Control	E2 Control	E2 E2
Breeding Tubercles	4±0.94[a]	5±2.0	4±1.6[a]	4±0.80[a]	7±1.9	5±2.2	6±1.4	12±1.4[b]
Dorsal Epithelial Pad	32.33 ±10.83[a]	43.54 ±20.64	38.09 ±20.81	30.80 ±11.86[a]	150.6 ±46.16[b]	39.61 ±20.98	123.6 ±24.48	59.59 ±23.04

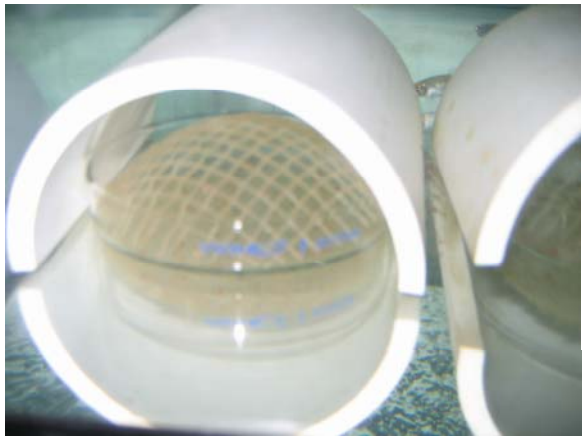


Image 3.1. Breeding substrate with egg tray intended to catch any non-adhered eggs from spawning events and maximize fecundity estimates.

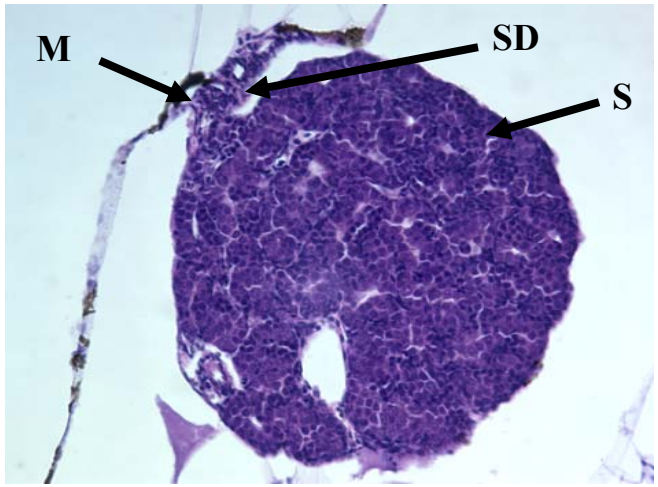


Image 3.2. Male F_1 Generation testicular cross-section (ControlControl) (40X).
M, mesentery; S, spermatogonia; SD, sperm duct.

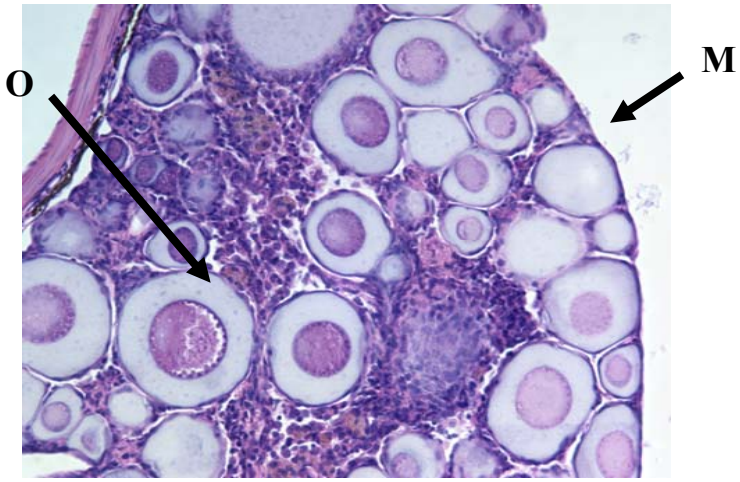


Image 3.3. Male F_1 Generation ovarian cross-section (ControlControl) (40X).
M, mesentery; O, stage III oocyte.

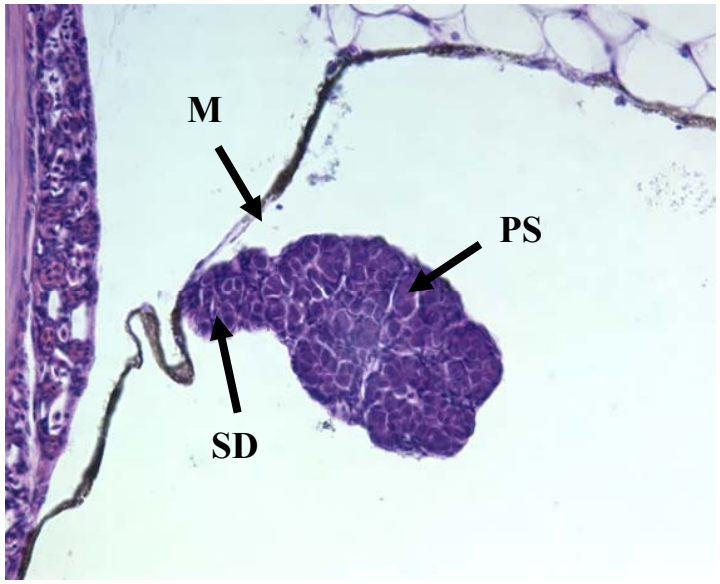


Image 3.4 Male F_1 Generation testicular cross-section (ControlE2(40X).
M, mesentery; PS, primary spermatogonia; SD, sperm duct.

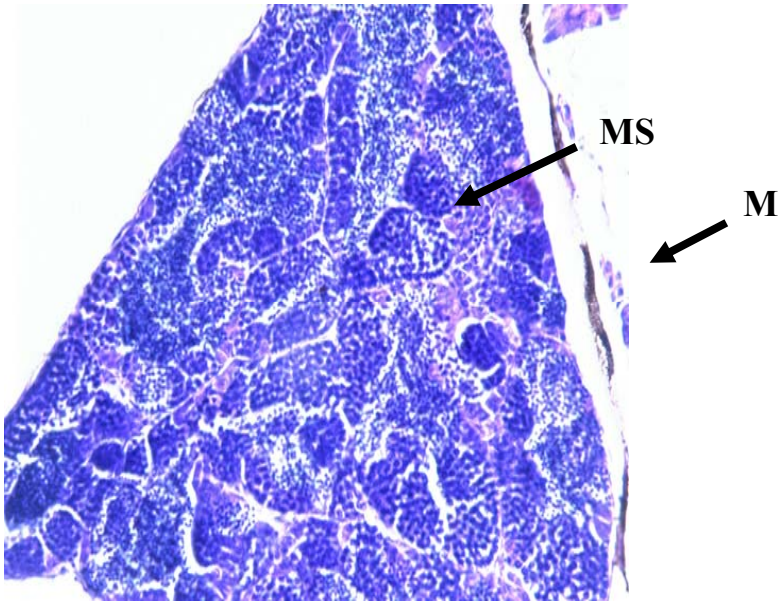


Image 3.5. Male F_1 Generation testicular cross-section (LowLow) (40X).
M, mesentery; MS, mature spermatozoa

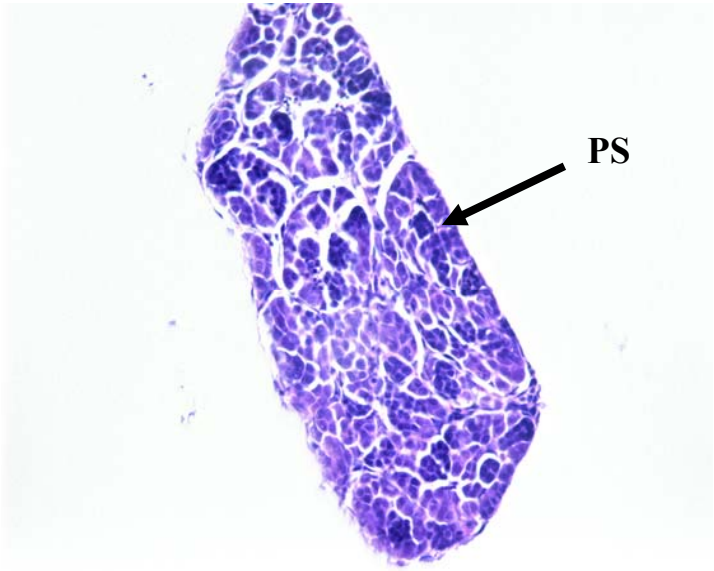


Image 3.6. Male F_1 Generation testicular cross-section (LowControl) (40X).
PS, primary spermatogonia.

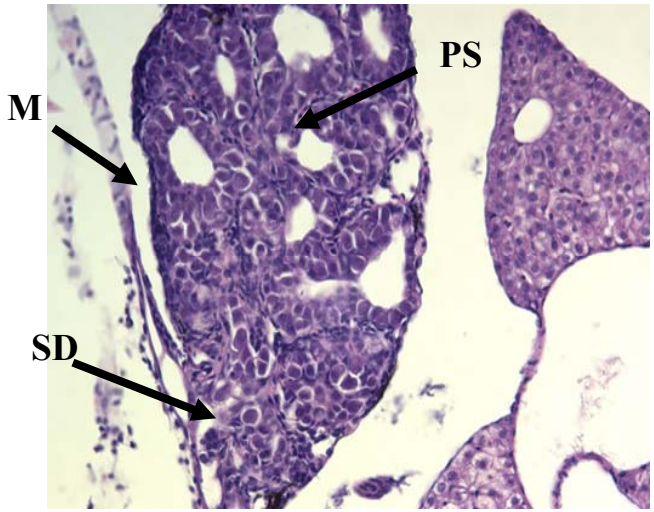


Image 3.7. Male F_1 Generation testicular cross-section (HighHigh) (40X).
M, mesentery; SD, sperm duct; PS, primary spermatogonia.

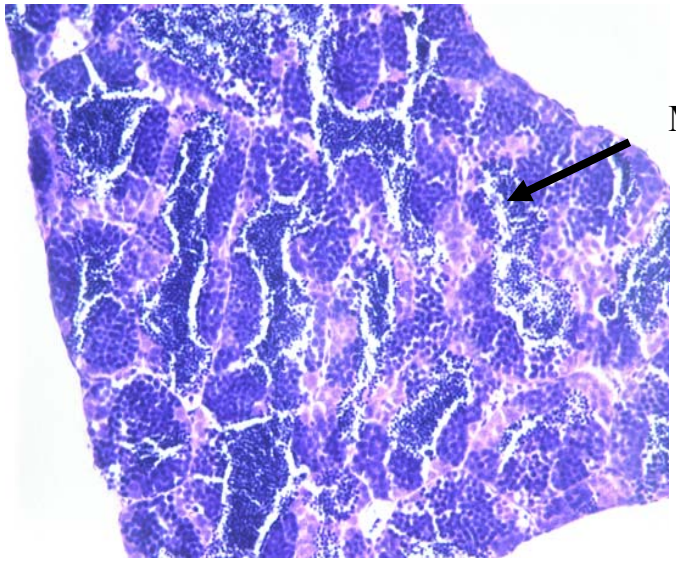


Image 3.8. Male F_1 Generation testicular cross-section (HighControl) (40X).
MS, mature spermatogonia.

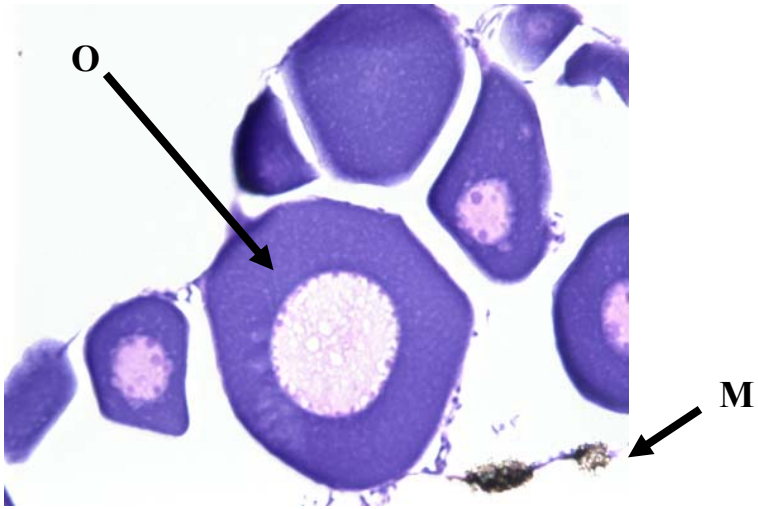


Image 3.9. Female F_1 Generation ovarian cross-section (HighHigh) (40X).
M, mesentery; O, stage III oocyte.

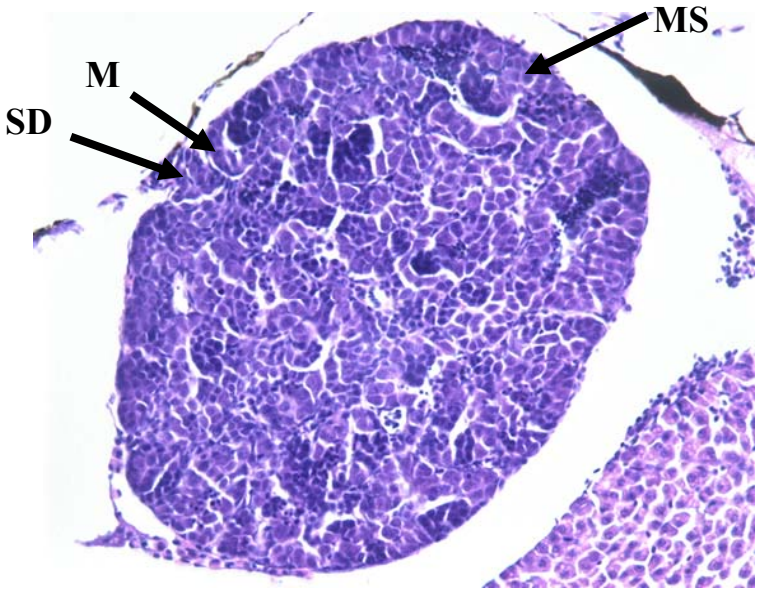


Image 3.10. Male F_1 Generation testicular cross-section (E2Control) (40X).
M, mesentery; SD, sperm duct; MS, mature spermatogonia.

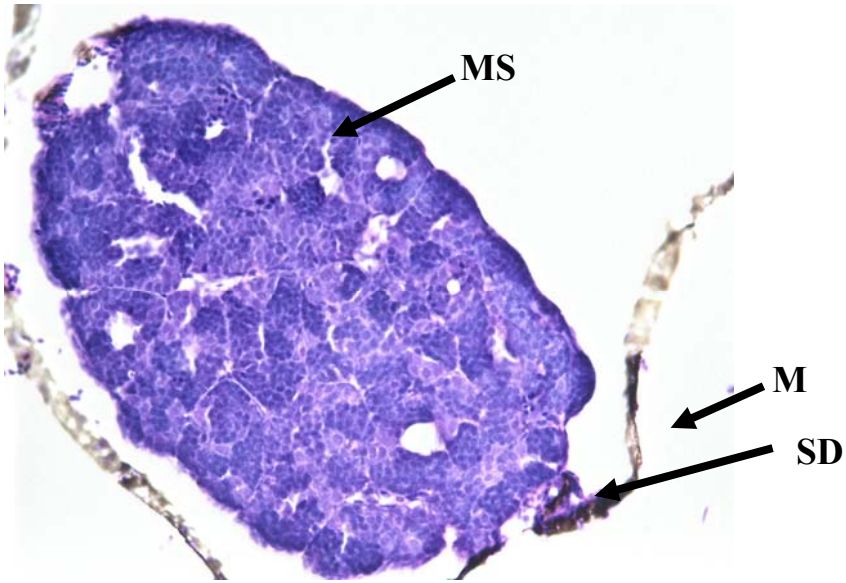


Image 3.11. Male F_1 Generation testicular cross-section (E2E2) (40X).
M, mesentery; SD, sperm duct; MS, mature spermatogonia.

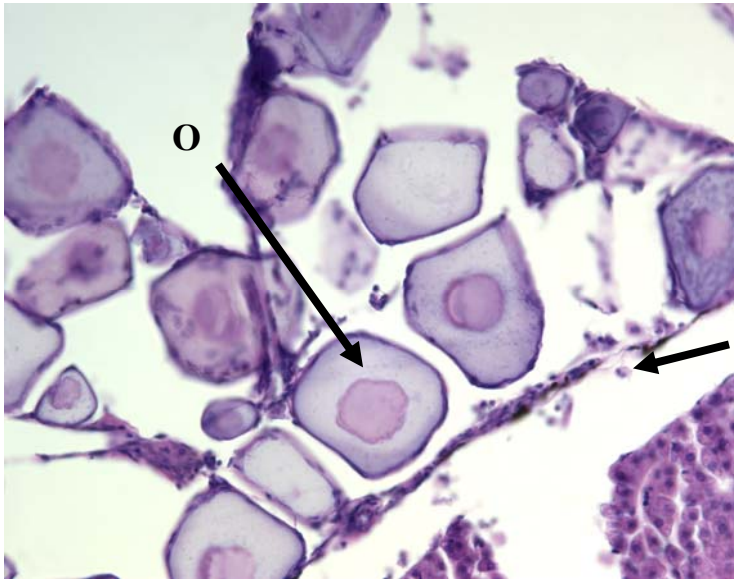


Image 3.12. Female F_1 Generation testicular cross-section (E2E2) (40X).
M, mesentery; O, stage III oocyte.

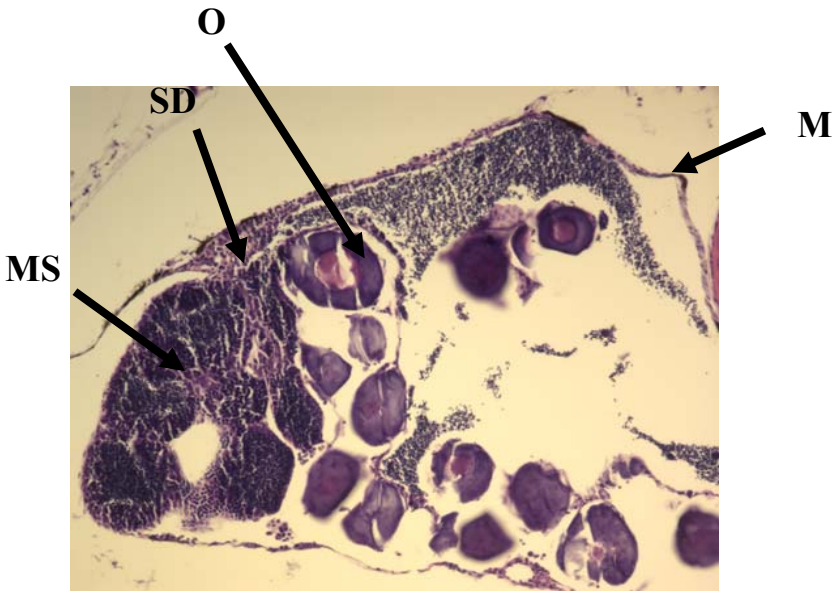


Image 3.13. Ovo-testis in presumptive male F_1 Generation LowControl (40X).
M, mesentery; SD, sperm duct; MS, mature spermatogonia; O, stage III oocyte.

Appendix I. ANOVA tables for Chapter 3 P_1 Generation analyses.

A. P_1 Generation cumulative fecundity

Source	Sum of Squares	df	Mean Square	F
Treatments	1.72×10^7	3	5.73×10^6	2.74
Error	6.66×10^6	20	1.33×10^5	
Total (Corrected)	8.38×10^7	23		

B. P_1 Generation fertility

Source	Sum of Squares	df	Mean Square	F
Treatments	0.02	3	0.008	0.60
Error	1.00	80	0.013	
Total (Corrected)	1.02	83		

C. P_1 Generation male breeding tubercles

Source	Sum of Squares	df	Mean Square	F
Treatments	5.85	3	1.95	0.30
Error	1190	186	6.40	
Total (Corrected)	1195.9	189		

D. P_1 Generation male dorsal epithelial pad

Source	Sum of Squares	df	Mean Square	F
Treatments	7921.4	3	2640.5	0.53
Error	8.81×10^5	176	5006.4	
Total (Corrected)	8.89×10^5	179		

E. P_1 Generation male interocular distance

Source	Sum of Squares	df	Mean Square	F
Treatments	0.04	3	0.01	0.15
Error	3.56	43	0.08	
Total (Corrected)	3.60	46		

F. P_1 Generation condition factor

Source	Sum of Squares	df	Mean Square	F
Treatments	1.60×10^{-7}	3	5.34×10^{-8}	3.39
Error	2.11×10^{-6}	134	1.57×10^{-8}	
Total (Corrected)	2.27×10^{-6}			

G. P_1 Generation gonadosomatic index (males and females)

Source	Sum of Squares	df	Mean Square	F
Treatments	355.3	3	118.4	0.87
Error	1.82×10^{4}	134	136.3	
Total (Corrected)	1.86×10^{4}	137		

H. P_1 Generation hepatosomatic index (males and females)

Source	Sum of Squares	df	Mean Square	F
Treatments	10.09	3	3.36	1.21
Error	372.4	134	2.78	
Total (Corrected)	382.5	137		

I. P_1 Generation 17β-estradiol (males)

Source	Sum of Squares	df	Mean Square	F
Treatments	0.03	3	0.01	0.19
Error	2.08	38	0.05	
Total (Corrected)	2.11	41		

J. P_1 Generation 17β-estradiol (females)

Source	Sum of Squares	df	Mean Square	F
Treatments	0.48	3	0.16	2.32
Error	5.68	83	0.07	
Total (Corrected)	6.16	86		

K. P_1 Generation testosterone (males)

Source	Sum of Squares	df	Mean Square	F
Treatments	0.82	3	0.27	2.41
Error	4.64	41	0.11	
Total (Corrected)		44		

L. P_1 Generation testosterone (females)

Source	Sum of Squares	df	Mean Square	F
Treatments	1.06	3	0.35	1.85
Error	16.07	84	0.19	
Total (Corrected)	17.14	87		

M. P_1 Generation E2/T ratio (males)

Source	Sum of Squares	df	Mean Square	F
Treatments	0.18	3	0.06	0.69
Error	3.44	40	0.09	
Total (Corrected)	3.62			

N. P_1 Generation E2/T ratio (females)

Source	Sum of Squares	df	Mean Square	F
Treatments	0.72	3	0.24	3.21
Error	6.12	82	0.07	
Total (Corrected)	6.83	85		

O. P_1 Generation vitellogenin

Source	Sum of Squares	df	Mean Square	F
Treatments	1.48×10^{11}	3	4.94×10^{10}	3.27
Error	6.25×10^{11}	41	1.49×10^{10}	
Total (Corrected)	7.74×10^{11}	44		

Appendix II. ANOVA tables for Chapter 3 F_1 Generation analyses.

A. F_1 Generation condition factor (females)

Source	Sum of Squares	df	Mean Square	F
Treatments	6.42×10^{-8}	7	9.17×10^{-9}	1.06
Error	1.92×10^{-6}	222	8.63×10^{-9}	
Total (Corrected)	1.98×10^{-6}	229		

B. F_1 Generation condition factor (males)

Source	Sum of Squares	df	Mean Square	F
Treatments	9.88×10^{-7}	7	1.41×10^{-7}	2.72
Error	7.72×10^{-6}	149	5.18×10^{-8}	
Total (Corrected)	8.71×10^{-5}	156		

C. F_1 Generation interocular distance (males)

Source	Sums of Squares	df	Mean Square	F
Treatments	3.84	7	0.55	1.39
Error	59.0	149	0.40	
Total (Corrected)	62.8	156		

D. F_1 Generation interocular distance (females)

Source	Sums of Squares	df	Mean Square	F
Treatments	1.07	7	0.15	1.24
Error	27.3	221	0.12	
Total (Corrected)	28.3	228		

E. F_1 Generation aromatase (males)

Source	Sums of Squares	df	Mean Square	F
Treatments	6.68	7	0.95	1.86
Error	75.60	147	0.51	
Total (Corrected)	82.28	154		

F. F_1 Generation aromatase (females)

Source	Sums of Squares	df	Mean Square	F
Treatments	8.53	7	1.22	3.75
Error	73.49	226	0.33	
Total (Corrected)	82.03	23		

G. F_1 Generation gonadosomatic index (males and females)

Source	Sums of Squares	df	Mean Square	F
Treatments	74.7	7	10.7	1.05
Error	1007.7	99	10.2	
Total (Corrected)	1082.4	106		

H. F_1 Generation hepatosomatic index (males and females)

Source	Sums of Squares	df	Mean Square	F
Treatments	14.1	7	2.02	1.54
Error	131.9	101	1.31	
Total (Corrected)	146.1	108		

I. F_1 Generation male breeding tubercles

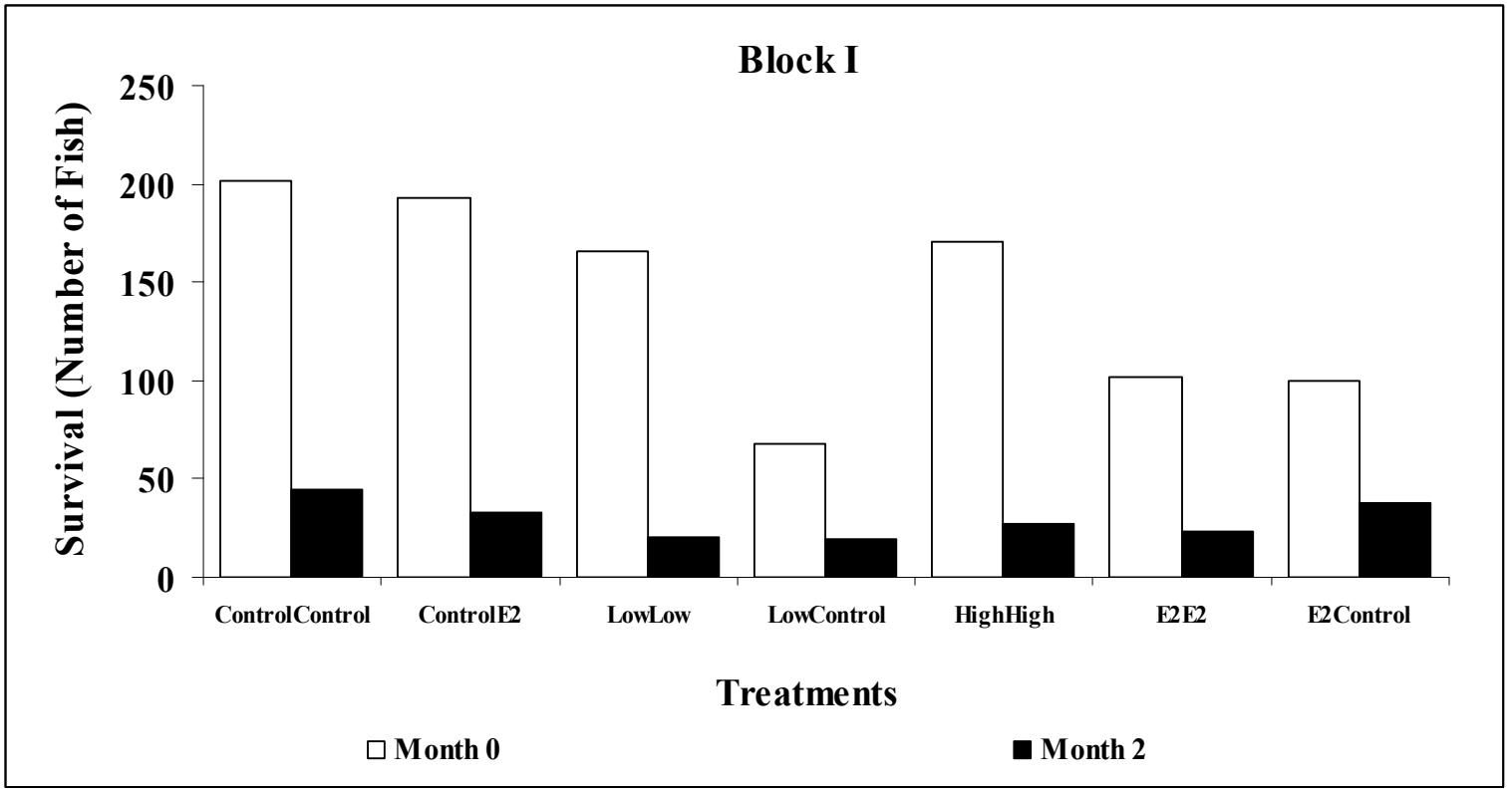
Source	Sums of Squares	df	Mean Square	F
Treatments	57.3	7	8.19	0.86
Error	847.7	89	9.52	
Total (Corrected)	905.0	96		

J. F_1 Generation male dorsal epithelial pad

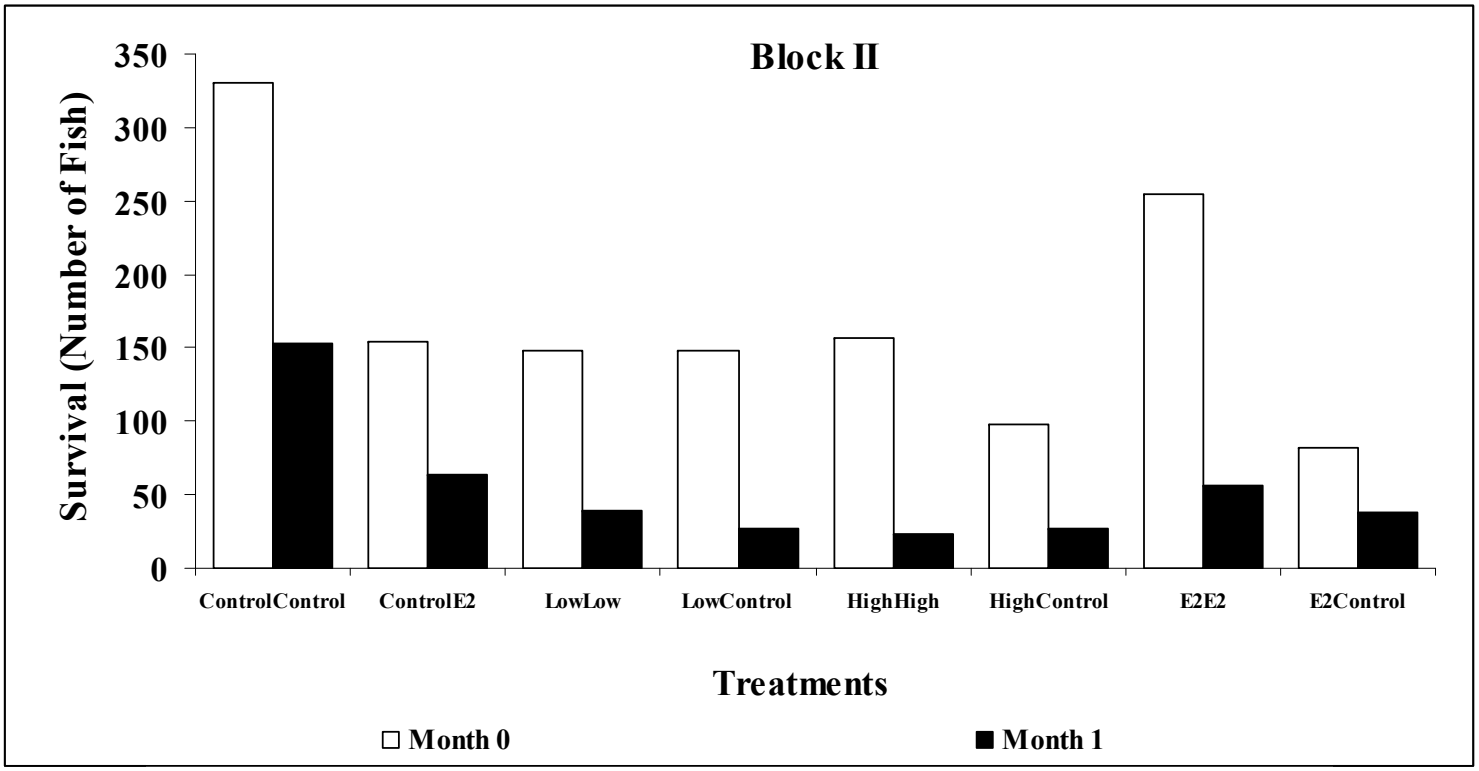
Source	Sums of Squares	df	Mean Square	F
Treatments	1.50×10^5	7	2.14×10^4	2.98
Error	6.39×10^5	89	7.18×10^3	
Total (Corrected)	7.89×10^5	96		

Appendix III. Graphs of densities (number of fish) of F_1 Generation fish compared to age in months.

A. Block I Survival (Number of Fish) v. Age in Months.



B. Block II Survival (Number of Fish) v. Age in Months.



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