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TEAM KERMIT

Research Proposal

University of Maryland: College Park

I pledge on my honor that I have not given or received any unauthorized assistance on this assignment.

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Abstract

Runoff from field-applied poultry litter contains considerable amounts of natural steroid hormones, such as estradiol, estrone, estriol and testosterone, which can potentially contaminate nearby ponds, streams and rivers. These hormones are of particular concern because extremely low concentrations (ng/L) of these endocrine disrupting compounds have been shown to adversely affect the reproductive biology of frogs and fish. We plan to test the potential effects of four concentrations (0-300 ng/L) of poultry litter associated hormones (PLAH) on *Xenopus laevis*' gonadal development, sex ratios, metamorphosis, growth and survival. The extended exposure (Day 5-120 post fertilization) will encompass the tadpole and juvenile stages, sexual development and metamorphosis. Simulated poultry litter runoff stock solutions will be allowed to degrade naturally throughout the study. Individual hormones will be quantified (GC-MS) weekly to assess the fate of PLAH. By conducting a long-term, environmentally relevant exposure to simulated poultry litter runoff, we should be able to determine the potential risk that PLAH might pose to amphibian reproductive development.

Evaluating the Effects of Long-Term Poultry Litter Associated Hormone Exposure to *Xenopus laevis*

The Delaware-Maryland-Virginia (Delmarva) area is one of the leading poultry producing regions in the United States. Increasing costs of synthetic fertilizers and the large surplus of poultry litter, chicken manure mixed with bedding, have resulted in the increased use of poultry litter as fertilizer in agricultural lands (Dutta, Inamder, Sims, & Collins, 2010). Poultry litter is an excellent natural fertilizer, containing high concentrations of nitrogen, phosphorous and potassium. It provides organic matter, which improves soil structure and nutrient and water retention (He, Endale, Schomberg, & Jenkins, 2009). However, poultry litter also contains high levels of endocrine disrupting chemicals (EDCs)—natural sex hormones including estradiol, estrone and testosterone—and trace amounts of heavy metals, such as lead, copper and arsenic (He, Endale et al., 2009).

The Delmarva poultry industry alone produces nearly 1.6 billion pounds of poultry litter per year (U.S. Department of Agriculture, 2002). Poultry litter is often applied to fields in spring and early summer, just prior to or after planting crops. The timing of poultry litter application coincides with amphibian breeding and larval development (Cogger, & Zweifel, 1998). During rainfall events, runoff from fields fertilized with poultry litter transports excess nutrients and hormones into adjacent ponds, lakes and streams (Finlay-Moore, Hartel & Cabrera, 2000). While the contamination threat posed by nutrient leaching from manure-treated fields is well recognized, the threat posed by the leaching of poultry litter associated hormones has received much less attention (Kjaer et al., 2007).

While much attention has focused on anthropogenic EDCs, such as pesticides, herbicides, PCBs, dioxins and alkyl-phenols, less attention and research has been directed toward natural

hormones in surface waters. The environmental load of natural hormones can potentially have a serious impact on aquatic organisms. The two main sources of hormone contamination include municipal wastewater effluents and runoff from soil amended with animal manures, including poultry litter. Shore, Harel-Markowitz, Gurevich, and Shemesh (1993) observed greater hormone concentrations in streams receiving runoff from poultry litter amended farms than those receiving effluent from sewage treatment plants.

Poultry litter contains appreciable amounts of natural estrogenic and androgenic hormones. Runoff and surface waters receiving agricultural runoff have been found to contain significant hormone concentrations. Finlay-Moore et al. (2000) found estradiol concentrations as high as 904 μg/kg in poultry litter. Shore and Shemesh (2003) measured estrogen and testosterone concentrations up to 533 and 670 μg/kg, respectively, in chicken manure. Nichols, Daniel, Moore, Edwards and Pote (1997) detected a maximum of 1,280 ng/L of estradiol in first-storm runoff water from plots amended with poultry litter. A thorough nationwide reconnaissance of streams impacted by animal waste showed that estradiol concentrations ranged from 0-200 ng/L and estrone concentrations ranged from 0-112 ng/L (Kolpin et al., 2002). Jenkins, Endale, Schomberg, and Sharpe (2006) found that runoff from freshly poultry manured fields had 38.7-196 ng/L of estradiol and 3.3-7.4 ng/L of testosterone. Kolpin et al. (2002) found estradiol concentrations as high as 120 ng/L in streams receiving agricultural runoff. Yonkos, Fisher, and Van Veld (2005) found that estradiol levels ranged from 19-75 ng/L in Maryland Eastern Shore streams and rivers.

Most of the laboratory and field studies evaluating the endocrine disrupting potential of poultry litter associated hormones have involved fish. Detection of the egg yolk protein precursor, vitellogenin, in males is a robust indicator of an exposure to an estrogenic stimulus

(Colborn, vow Saal, & Soto, 1993) and has been shown to be predictive of subsequent reproductive and histopathological effects (Tyler, Jobling & Sumpter, 1999). Lahnsteiner, Berger, Kletzl, and Weismann (2006) reported that estradiol concentrations of 1 ng/L drastically reduced sperm fertility and induced vitellogenin in male rainbow trout. Seki, Yokota, Maeda, and Kobayashi (2005) reported that exposure to 8.7 ng/L of estradiol caused intersex in medaka (*Oryzias latipes*). Routledge et al. (1998) demonstrated that estrone and estradiol exposure led to the demasculinization of male rainbow trout (*Oncorhyncus mykiss*) at a 25 ng/L concentration.

Over the last decade, research has indicated that amphibians are particularly sensitive to EDCs (MacKenzie, Berrill, Metcalfe, & Pauli, 2003). The majority of the amphibian research has examined the effects of exogenous EDCs like herbicides and pesticides, manufacturing chemicals, plastic products such as bisphenol A, and surfactants, such as octylphenol, and nonylphenol (Colborn et al., 1993; Hayes & Collins et al., 2002; Huang, Matthews, Fertuck, & Zacharewski, 2005; Levy, Lutz, Kruger, & Kloas, 2004; Mackenzie et al., 2003). In many of these studies, estradiol was used as a positive control, causing gonadal abnormalities and altered sex ratios in leopard frogs (*Rana pipiens*) at concentrations as low as 1 ng/L. Wolf et al. (2010) have performed the most comprehensive study on the effects of estradiol exposure on amphibian gonadal development. They found that an estradiol concentration of 200 ng/L significantly altered sex ratios and caused gonadal abnormalities in *Xenopus laevis*. Most research, like the study by Wolf et al. (2010) is done with pure chemicals, in this case, estradiol. Very little amphibian research has been conducted on the complex mixture of natural hormones associated with poultry litter runoff. We will refer to these hormones as PLAH (poultry litter associated hormones).

We plan to investigate the potential effects of environmentally relevant concentrations of PLAH on amphibian development. We will use *X. laevis* as our model organism. Although not native to the Delmarva area, *X. laevis* has been designated as a model aquatic anuran species by the Endocrine Disrupting Screening and Testing Assessment Committee of the U.S. EPA. We will use a negative control and three concentrations of simulated poultry litter runoff based on the concentration of estradiol. We plan to look for gonadal changes in frogs over a 115 day exposure time. Additionally, we will allow the poultry litter solutions to degrade naturally over the 115 days of the study to determine the fate of the hormones over time. We will monitor gonadal development, sex ratios, mortality, time until metamorphosis and growth in *X. laevis*.

Literature Review

Poultry Litter

Chickens in the United States secrete approximately 2.7 metric tons of estrogens per year (Hanselman, Graetz, & Wilkie, 2003). Farmers use poultry litter as pre-planting fertilizer to enrich the soil since it provides high levels of macronutrients and micronutrients. Previous research shows that poultry litter increases nitrogen and phosphorus levels in water, but has not focused on the effect(s) of poultry litter on amphibians' reproductive systems (Curtis, Kingery, Cox, & Liu, 2010; Haggard, Delaune, Smith, & Moore, 2005; He, Honeycutt, Tazisong, Senwo, & Zhang, 2009). Although many consider poultry litter as a natural substitute for many synthetic fertilizers, it has a harmful impact on the environment as a major source of estradiol in agricultural runoff (Moore, Daniel, Sharpley & Wood, 1995).

During heavy rainfall after poultry litter application, runoff containing nutrients and hormones from poultry litter is washed into major bodies of water and thereby distorts the normal hormonal balance (Moore et al., 1995; Lange et al., 2002). Moreover, excessive

application of poultry litter on fields can lead to increased concentrations of nitrogen, phosphorous, pathogenic microorganisms, chemicals, and hormones in groundwater and down-stream surface water (Moore et al., 1995). Kolpin et al. (2002) have shown that the increase in hormone levels in water has significantly affected the reproductive systems of aquatic organisms. Because poultry litter has been shown to contain high levels of estradiol and is one of the most commonly used agricultural fertilizers, it is crucial to study the effects of poultry litter on aquatic organisms.

Steroid Hormones

EDCs ultimately affect the endocrine system and its functions by mimicking, antagonizing, or interfering with the biosynthesis and biodegradation of natural hormones. (Burkholder et al., 2007; Coulcci, Bork & Topp, 2000). Specifically, Wolf et al. (2010) linked EDCs to partial or complete conversion from the male to the female phenotype as defined by: intersex, testicular oocytes, ovotestes, and hermaphroditism in *X. laevis* and in other species of frogs. They also found altered sex ratios, retarded gonadal development, discontinuous gonads, and pigmentation changes (Glossary).

Estradiol is the most bioactive of the natural estrogens (Finlay-Moore et al., 2000). In the environment, estradiol has been found at levels well above those shown to stimulate cellular response (Burkholder et al., 2007). In a national reconnaissance, Kolpin et al. (2002) found that, although there were lower concentrations of reproductive hormones compared to other organic wastewater contaminants, hormones including estradiol had notable effects on the ecosystem due to greater toxicity; even an exposure less than 1 ng/L of estradiol produced deleterious effects in aquatic species. With an increase in estradiol exposure, Wolf et al. (2010) observed a significant increase in the number of frogs with ovaries, in addition to an increase in mixed sex frogs.

Estrone is another EDC that is detrimental to the endocrine systems of aquatic organisms due to its similar conjugated structure (Hanselman et al., 2003). Estradiol has a tendency to decompose readily to estrone, another biodegradable compound (Coulcci et al., 2001). It has been predicted that estradiol found in manure, in typical agricultural conditions, will have maximized contact with soil and microbes leading to accelerated degradation (Coulcci et al., 2001).

Further study on estradiol is necessary because of its severe effects on the reproductive systems of organisms. Additionally, we will measure other natural estrogens and androgens, such as estriol, testosterone and dihydrotestosterone, to assess their potential contributions to detrimental effects on gonadal development. Measuring these androgens and estrogens will also provide valuable information on the degradation of natural hormones in poultry litter runoff.

Relevant hormone concentrations.

Wolf et al. (2010) performed studies on the effects of estradiol on *X. laevis* with four concentrations: 0; 200; 1,500; and 6,000 ng/L. Through their study, Wolf et al. (2010) determined 200 ng/L as the approximate concentration where 50% of genotypic males would covert to phenotypic females. At the end of their experiment, they found that complete feminization and severe morphologic effects resulted from an exposure to concentrations greater than 200 ng/L. Additionally, Wolf et al. (2010) hypothesized that frogs would have a higher prevalence of reproductive abnormalities, rather than complete feminization, at concentrations lower than 200 ng/L. We will build upon their research by studying synergistic effects of PLAH.

After reviewing existing research, we established an environmentally relevant range of concentrations for our study. Nationwide reconnaissance data by the U.S. Geological Survey showed that estradiol and estrone concentrations were respectively 200 and 112 ng/L in a

network of 139 streams impacted by animal wastes (Hanselman et al., 2003). Both Hanselman et al. (2003) and Finlay-Moore et al. (2000) found that concentrations can range from 20 ng/L to 2,530 ng/L. Another study found that estradiol concentrations ranged between 6 and 66 ng/L in five spring samples from mantled karst aquifer systems of northwest Arkansas (Peterson, Davis, & Orndorff, 2000). Yonkos et al. (2005) found that estradiol concentrations ranged from 19 ng/L to 75 ng/L in Maryland Eastern Shore streams and rivers that receive agricultural runoff.

Analysis of hormone concentration.

We will be working with concentrations of PLAH in the ng/L concentration range. Because we want to determine the decomposition of PLAH over time, it is critical that our analysis of the concentration is very accurate. Thus we will use GC-MS (gas chromatography-mass spectrometry) for our analysis. GC-MS or LC-MS (liquid chromatography-mass spectrometry) is superior to immunoassays like ELISA (Enzyme-linked immunosorbent assay) because of its accuracy and specificity for identifying individual chemicals (Dutta, Inamdar, Tso, Aga, & Sims 2010). For instance, ELISA analysis generally yields higher estrogen concentrations because of its lack of specificity when identifying organic compounds (Dutta, Inamdar, Tso et al., 2010). Finally, immunoassays must be independently confirmed by GC-MS methods, making them an unsuitable choice in this study (Ingerslev, Halling-Sørensen, 2003).

Xenopus laevis

Of all frog species, *X. laevis* is commonly used for EDC research (van Wyk, Pool, & Leslie, 2003). Although *X. laevis* is not a native species to the greater Chesapeake Bay area, previous studies show that this frog species can be used as a model organism for toxicity in other frog species. *X. laevis* is a model organism in amphibian EDC research because researchers can easily induce reproduction, which yields a large number of offspring (Bevan, Prasad, &

Henderson, 2001). Using a large number of offspring from the same breeding pair lends statistical significance and controls for a possible confounding variable in our experiment. In particular, amphibians are an appropriate test subject since these organisms breed and develop in standing water that could contain runoff contaminated by poultry litter (Wolf et al., 2010). The entire *X. laevis* life cycle is aquatic so the exposure to poultry litter will be maximized (Bevan et al., 2001). This effect is further amplified by the semipermeable skin and hormone regulated development of *X. laevis.*

Abnormal larval development and metamorphosis.

Prior research has found that between stages 52-54 *X. laevis* undergoes normal sexual differentiation (Hayes & Stuart et al., 2006). Hayes & Stuart et al. (2006) chose to apply estradiol and atrazine, a pesticide, at stage 50, approximately 6 days before sexual differentiation in order to expose the *X. laevis* to chemicals well before and throughout their critical period of reproductive development. *X. laevis* normally completes metamorphosis at stage 66 or about 55 to 75 DPF (Wolf et al., 2010; Glossary). Wolf et al. (2010) found that at the completion of metamorphosis, there was a wide variation in the degree of overall gonadal development in both males and females. Although identification of abnormal gonadal tissues can be seen at this point, we wish to extend the study to 120 DPF to allow the frogs time to grow, and for further differentiation and development of gonadal tissue.

Gonadal histology.

Sexual differentiation and mutation must be observed by performing a gonadal histology (Orlando et al., 2004; Wolf et al., 2010). Orlando et al. (2004) performed a gonadal histology to examine the effects of EDCs released from cattle feedlots on the development of fathead minnows and to determine whether the subjects reached sexual maturity. To perform a gonadal

histology, Wolf et al. (2010) prepared gonad tissues on glass slides using Permount solution (Fisher Scientific), and stained the slide with hemotoxylin and eosin to differentiate between various gonadal tissues. These studies can elucidate any unusual variations in internal organ size, color, or structure. From these histological studies, Wolf et al. (2010) were able to find that, at the completion of metamorphosis, there appeared to be a wide variation in the degree of overall gonadal development in both males and females. Lutz et al. (2008) also described histology as one of the accepted methods to evaluate effects of EDCs on the development and sexual differentiation of *X. laevis.* From this study, which served to evaluate various sexual differentiation tests, the researchers found that histological studies can determine the presence of intersex and mixed sex characteristics (Lutz et al., 2008; Glossary).

Observations and endpoints.

Wolf et al. (2010) on the effects of estradiol on the sexual differentiation of *X. laevis*, monitored the tadpoles' general health and swimming behavior (for example, whether the tadpoles swam in circles) and observed morphological changes and any abnormal feeding behaviors. Wolf et al. also recorded the number of deceased tadpoles and the number of tadpoles that completed metamorphosis. Furthermore, they measured the snout-vent length and wet weight (Glossary). Completion of metamorphosis was delayed by 2-3 days after *X. laevis*' exposure to estradiol.

EDC Exposure in Humans

The effects of EDCs are not limited to aquatic species; they can also have detrimental effects on human health. Humans are exposed to estrogenic compounds in food, plastics, pesticides, and other chemicals (Harrison, Holmes, & Humfrey 1997). Estradiol can seep into groundwater or infiltrate surface water via runoff, and eventually contaminate bodies of water or

drinking water (Orlando et al., 2004). Around the world, male reproductive health has been on the decline. The USA, Finland, Denmark, and the UK have had higher rates of testicular cancer and prostate cancer in recent years (Harrison et al., 1997). There is evidence of lower semen quality including lower sperm count and decreased motility (Damstra, 2002). Another study found that human fetal exposure to high levels of estrogenic compounds can lead to an increased risk of male gonadal disorders (Toppari et al., 1996).

Methodology

We need quantitative data that describes poultry litter's effects on *X. laevis* metamorphosis and sexual development. To determine the effects of poultry litter, we will randomly divide frogs into three different experimental groups and one control group. We will have five replicates of each group, which will consist of 25 frogs. We will then expose the frogs in the experimental groups to different concentrations of PLAH based on the concentration of estradiol. We will perform a gonadal histology on the frogs 120 DPF to determine the extent to which, if any, poultry litter affects the sexual development of frogs. We chose 120 DPF because this gives the additional time to grow after metamorphosis, allowing for greater differentiation of gonadal tissue and greater ability to distinguish gonadal abnormalities.

Research Design

Most studies adhered to methods set out in the *Standard Guide for Conducting Whole Sediment Toxicity Test with Amphibians* published by the American Society for Testing and Materials (American Society of Testing and Materials [ASTM], 2006). This study will be performed in the laboratory. The laboratory setting allows us to control for extraneous variables like temperature, amount of food, frog density and natural predators that would be impossible to control in the field. Furthermore, in the field we would be unable to ensure the runoff contained

varying concentrations of PLAH, with no other contaminants. These other contaminants could cause problems in metamorphosis and abnormalities in the frogs' reproductive organs. Additionally field testing would introduce genetic variation among the frogs being tested. While this cannot be fully avoided, we are minimizing this by using the offspring from one breeding pair. Laboratory testing will limit these extraneous variables, so we can determine the potential effects of PLAH alone on frog development.

Acquisition of embryos.

We will obtain *X. laevis* embryos from breeding colonies at the University of Maryland - Wye Research and Education Center (UMD-WREC). We will maintain the adult colonies in flow through (4 replacement volumes per day) circular polyethylene aquaria (0.91 m I.D. x 0.36 m high) with a water depth of 10 cm. Each aquarium can contain a maximum of 10 adults. UMD-WREC non-chlorinated deep well water held at 23.0 ± 0.5°C will serve as a culture medium. We will feed breeding frogs daily with Xenopus Express Premium Floating Food. We will keep the colony under a photoperiod of 16 h light: 8 h dark. We will inject 400 and 800 I.U. of human chorionic gonadotropin (HCG) in the dorsal lymph sac of the males and females, respectively, during the dark cycle in order to induce breeding (Glossary). Amplexus will occur 4-6 hours after injecting HCG (Glossary); egg deposition will occur 9-12 hours following HCG injection.

Tadpole selection.

X. laevis embryos will be maintained at UMD-WREC in non-chlorinated deep well water until they reach stage 45-46 or 4 DPF. Mr. Steve Turley will bring up a large number of tadpoles from one breeding pair to our laboratory in Room 1519 in the Biochemistry Building. The

embryos will be transported in well water that has been saturated with oxygen in sealed polyethylene containers.

The embryos will be allowed to acclimate in our on-campus laboratory for 24 hours prior to the start of the test. At 5 DPF, embryos will be examined for viability and proper staging using a dissecting microscope. A subset of ten embryos will be preserved in ethanol to verify the starting age of test organisms. The embryos will then be randomly loaded into each test replicate. Test embryos will be obtained from a single breeding pair to eliminate genetic variability.

Preparation of poultry litter.

We will obtain a large sample of poultry litter (100 lb.) from an Eastern Shore concentrated animal feeding operation (CAFO or chicken house). The sample will be brought to the UMD-WREC and kept sealed at 4°C in the dark. The poultry litter sample will be thoroughly homogenized. Two aqueous subsamples will be shipped to the University of Buffalo for hormone analysis. Three simulated poultry litter runoff test solutions will be prepared based on the estradiol concentrations measured in the poultry litter. The stock solutions will be prepared by mixing varying amounts of poultry litter and diluting with UMD-WREC well water. The stock solutions will be stored in 55-gallon drums at room temperature. Stock solutions will be allowed to degrade naturally, with only gentle aeration and circulation being applied to keep the solutions homogenized. Adequate amounts of control well water and the three poultry litter stock solutions will be transported each week from UMD-WREC to the on-campus laboratory to perform the twice per week test solution renewals.

Primary endpoints.

Our primary endpoints are gonadal development and abnormalities, sex ratios, and mortality. We will closely examine gonadal development in *X. laevis*. Histological analyses will

reveal any gonadal abnormalities the frogs may have developed after exposure to poultry litter. Additionally, as concentrations of poultry litter increase, we will be monitoring the ratio of female to male frogs. Altered sex ratios and abnormal gonadal development could significantly reduce fertility and decrease or prevent successful breeding, causing population numbers to decrease via diminished recruitment of juveniles. Mortality is another important endpoint. Individuals must complete metamorphosis, survive to sexual maturity and successfully breed for a population to thrive and grow. Additionally, high concentrations of chemicals such as ammonia, phosphorus, and heavy metals such as copper and zinc are potentially toxic to frogs (He, Honeycutt et al., 2009).

Secondary endpoints.

Our secondary endpoints are time to metamorphosis, snout-vent length, and wet weight. Time to metamorphosis is an indicator of eventual survival. Metamorphosis usually occurs between 55 and 75 DPF, but with an increase in poultry litter concentration, the onset of metamorphosis may be delayed. There are also a number of benefits for amphibian larvae reaching a larger mass, including greater overwintering success, greater survival to first reproduction, and earlier reproduction (Semlitsch, Scott, & Pechmann, 1988). Larger females can carry more eggs, and larger males often gain access to a greater number of females during breeding, leading to increased reproductive success (Bridges, 2000).

Testing setup.

UMD - WREC non-chlorinated deep well water will serve as the control water and the dilution water for poultry litter. This study will use 5 replicates of 25 tadpoles, which will be exposed to each of the following environmentally relevant treatments: 1) control water (0 ng/L), 2) 15 ng/L, 3) 60 ng/L, and 4) 300 ng/L of estradiol. We will maintain each replicate in a 5-

gallon aquarium (16”x11”x8”) filled with 4 L of control water or poultry litter solution initially. We will increase the test solution volume to accommodate *X. laevis* growth.. We will maintain the initial water volume of 4 L for the first four weeks; thereafter, we will increase water levels by 1 L every other week, until we reach a maximum of 8 L. We will maintain a volume of 8L for the remainder of the study. We will maintain the temperature at 23 ± 2°C with a 16 hour light: 8 hour dark cycle. We will perform 25-33 percent water replacements twice per week. Debris, fecal matter, excess food and dead organisms will be removed on a daily basis. Gentle aeration (1 bubble/second or enough to maintain a dissolved oxygen level of 5-6mg/L) will maintain adequate water quality by reducing ammonia levels. We will measure dissolved oxygen and pH daily, and conductivity, alkalinity, hardness, nitrates, and ammonia weekly. A study by Lutz et al. (2008) noted optimum parameters for water quality include pH (optimally between 7.9 and 8.3), ammonia (between 0 and 0.35 mg/L), nitrate (between 0.04 and 2.29 mg/L), hardness (between 90 and 130 mg $CaCO_3$/L), alkalinity (between 133 and 172 mg $CaCO_3$/L), and conductivity (726-817 µg S/cm). Starting at test initiation, subsamples from each replicate will be composited for each of the three poultry litter test solutions. Each week for the first month of the test, the three composite samples will be shipped off for hormone analysis. After the first month of the exposure, composite samples will be collected and shipped for analysis on a bi-weekly or monthly basis.

Starting at 5 DPF, tadpoles will be fed twice daily with Xenopus Tadpole Powder (Xenopus Express, Inc., Brooksville, FL). Survival and behavioral observations will be made daily. Metamorphosis normally occurs between 55-75 DPF. As the organisms complete metamorphosis, a screen will be placed between metamorphosed (juvenile) frogs and tadpoles to prevent predation, while allowing water flow and maintaining the exposure. Time to complete

metamorphosis will be noted for each organism. Juvenile frogs will be fed with Xenopus FFF (floating frog food)(Xenopus Express Inc., Brookville, FL) once per day. The screen will be removed when all frogs have completed metamorphosis.

Histology and preservation.

The frogs are classified as juveniles from the time they complete metamorphosis until they are sexually mature. At 120 DPF, we will sacrifice the frogs by immersion in MS-222 (Glossary). The frogs will then be rinsed, and snout vent length and wet weights will be measured. We will photograph every frog so we have a record of any external macroscopic abnormalities. The frogs will then be preserved in 70% ethanol. A single, mid-line incision will be made to allow for preservation of the internal organs. We will dissect the frogs and make gross observations of gonads both macroscopically and microscopically using a dissecting microscope. We will excise and preserve the gonads in Bouin's solution. If financially feasible, we will process, paraffin-embed, and microtome gonad tissue for preparation of stained slides.

Pilot study.

We will perform a preliminary study to assess and amend test maintenance and protocols, water quality (specifically ammonia levels), tank density, juvenile frog preservation, feeding rates, and the type of food to use (floating vs. sinking). We will determine the percent water change required to maintain adequate water quality with minimal disruption to the organisms. We will test to find a feeding rate that will maximize growth and development, while maintaining adequate water quality and minimizing waste. Finally, we seek to gain experience with performing gonadal histology of small *X. laevis* frogs.

Anticipated Results

We anticipate that we will find changes in the reproductive system of *X. laevis.* The primary indicators of changes in frog reproductive development will be gonadal development, sex ratios, and mortality. The secondary endpoints are time to metamorphosis and growth. We anticipate that tadpoles exposed to poultry litter will have greater mortality rates and that the metamorphosis time of tadpoles will increase (Wolf et al., 2010). We also anticipate that there will be an increase in snout-vent length and wet weight as a result of the increased metamorphosis time (Wolf et al., 2010). We anticipate that at higher concentrations of poultry litter, we will find higher percentages of intersex characteristics (Wolf et al., 2010). We also anticipate that as concentrations of poultry litter increase, so will the phenotypic sex ratios of female to male frogs (Wolf et al., 2010).

Statistical Analysis

While most of our data will be quantitative, we also have qualitative which will need to be quantified. Therefore, we will assess the amount of male tissue in the ovaries and the amount of female tissue in the testes with percentages. We will have percentage ranges from 0 – 24, 25 – 49, 50 – 74, and 75 – 100. If the percentage falls between 0-24 it will be ranked a 4, 25-49 a 3, 50-74 a 2, and 75-100 a 1. In order to evaluate the statistical significance of our results we will use Shapiro-Wilk's test, Barlett's test, Dunnett's test, Steel's Many-One Rank test, and Wilcoxon Rank Sum Test with Bonferroni Adjustment. Using Shapiro-Wilk's test, we will test all data for normality. We will then use Barlett's test to test for the homogeneity of variance. In order to test the null hypothesis, we will use Dunnett's test which includes ANOVA. ANOVA will be used to determine error, which will be used in further comparison tests to compare the control and experimental groups. We will further analyze the groups that do not meet the normality or

variance, using nonparametric statistics such as Steel's Many-One Rank Test or the Wilcoxon Rank Sum Test with Bonferroni Adjustment. These statistical tests will have an alpha level of 0.05 and will use ToxCalc (TTS, 2006).

Extraneous/Confounding Variables

Not only does poultry litter contain known EDCs such as estradiol, but it also contains heavy metals such as zinc and copper in excess (He, Endale et al., 2009). This is relevant because the other chemicals in poultry litter can have effects on the endocrine system, potentially affecting gonadal development (Bicchi et al., 2009). However, we are looking at the effects of poultry litter hormones as a whole on the reproductive systems of frogs, not solely estradiol. Though not all poultry litter will have the same ratio of PLAH, we chose to base our poultry litter runoff solutions based on estradiol because it has been shown to be the main cause of reproductive deformations (Burkholder et al., 2007; Coulcci et al., 2001). Further, our study is still valid because the concentrations of the other chemicals will decrease linearly in proportion to the amount of water used in the dilution.

Conclusion

Poultry litter management, application, and runoff are major issues in agriculture, that have far-reaching potential consequences for neighboring ecosystems. Elevated levels of these hormones are already known to pose a threat to aquatic wildlife. Our research will also address the potential risks that municipal and industrial waste poses to nearby life, including humans (Dorabawila, & Gupta, 2004). These natural and synthetic hormones are found in bioactive concentrations in effluents from wastewater treatment plants. In humans, increased EDC concentration in the environment may be linked to higher breast cancer rates, higher prostate cancer rates, higher testicular cancer rates, and decreased sperm counts (Wolff et al., 1995).

Closely monitoring the effects of environmentally relevant concentrations of contaminants in poultry litter runoff using a model species such as *X. laevis* will allow us to make specific recommendations for the future. The results could highlight the potential dangers of poultry litter hormones as a major source of EDCs in neighboring environments. Some research has been done on amendments to poultry litter, such as the addition of alum, aluminum sulfate. Nichols et al. (1997) found that the addition of alum to poultry litter greatly reduced levels of EDC runoff into the environment. Our research and further study on solutions for the future can aid federal environmental agencies, such as the EPA, in determining the extent and necessity of future regulations. If the usage of poultry litter does lead to severe irreversible effects, there will be a definite need for stricter regulations on its widespread use in agricultural lands.

Appendices

Timeline

	2010		2011			
	Fall	Winter	Spring	Summer	Fall	Winter
IACUC Proposal						
Develop Website						
Update Website						
Pilot Study & Histology Practice						
Thesis Proposal						
Grant Application & Submission						
Find lab for water analysis						
Breed Frogs						
Order Materials						
Set up lab space						
Conduct Research						
Analyze Gonadal Tissue						
Poster & Undergraduate Research Day						
Write Thesis						
Present & Defend Thesis						
Revise Thesis for Publication						

	2012				2013	
	Spring	Summer	Fall	Winter	Spring	Summer
IACUC Proposal						
Develop Website						
Update Website						
Pilot Study & Histology Practice						
Thesis Proposal						
Grant Application & Submission						
Find lab for water analysis						
Breed Frogs						
Order Materials						
Set up lab space						
Conduct Research						
Analyze Gonadal Tissue						
Poster & Undergraduate Research Day						
Write Thesis						
Present & Defend Thesis						
Revise Thesis for Publication						

Budget

Item	Unit Price	Quantity	Total
Commercial Adult Xenopus Food	$20	1 bag	$20
Commercial Tadpole Xenopus Food	$20	1 bag	$20
Tank Maintenance/Upkeep Items	N/A	N/A	$210
Histology Materials	N/A	N/A	$500
Poultry Litter	N/A	1 pile	$50
Poultry Litter Sample Analysis	$300	25	$7500
pH meter & dO_2 meter	$500	1	$500
Lighting	$10	20	$200
		Total	$9,000

Glossary

Amplexus – pseudocopulation where the male fertilizes the eggs while grasping the female with his front legs. Fertilization of the eggs is external

Bouin's solution – frequently used to preserve soft/fragile structures

Critical period of reproductive development – the time period in which the reproductive organs are developing and the organism is particularly sensitive to environmental factors

Discontinuous gonad – multiple gonad or segmented gonad, discrete subunits with obvious gonadal tissue separated by thin pieces of connective or non-gonadal tissue

Dorsal lymph sac – in the frogs' back, roughly in the middle, lengthwise – see picture

DPF – days post fertilization

Feminization (morphological) – partial or complete conversion from the male to the female phenotype

Hermaphroditism – form of intersexuality in which both male and female gonadal tissues are present in the same individual

Human chorionic gonadotropin – hormone that induces pregnancy

Intersex – assessed as ovarian and testicular tissue in the same individual as separate gonads (left/right)

Microtome – apparatus used to slice very thin sections for analysis with microscope

Mixed sex – defined as the co-occurrence of both ovarian and testicular tissue in a single gonad.

MS-222 – (tricane mesylate) white powder commonly used for sedation or euthanasia

Ovotestes – gonadal tissue that is more than 30% female

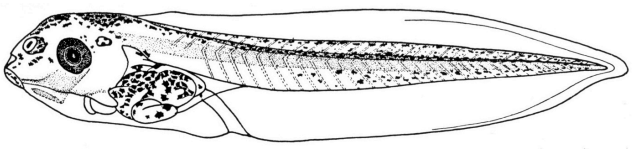
PLAH – poultry litter associated hormones

Snout vent length – distance from the tip of the snout to the anus

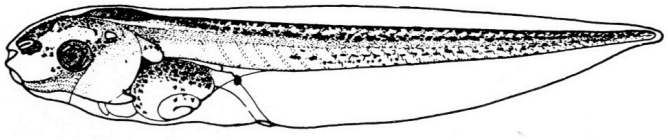
Stage 45-46 tadpole – see image below

Testicular oocytes – Testicular tissue with single or few oocytes, female gametocyte/egg before fertilization

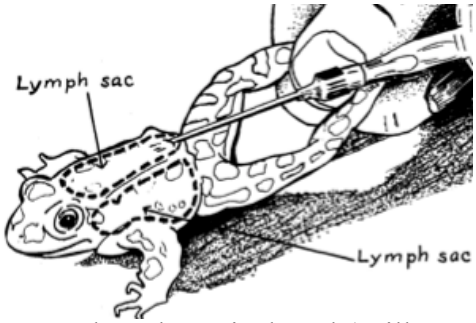
Wet weight – weight of frog without drying



Stage 45 tadpole



Stage 46 tadpole



Top lymph sac is dorsal (Miller, D.F., & Wiltberger, P.B. 1948)

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