Effects of 17α-ethynylestradiol on the Anatomical Development of *Xenopus laevis*

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Abstract

Xenopus laevis spends its entire life cycle in an aquatic environment. Consequently, it may be exposed to many water-based compounds, be they natural or synthetic. Concern has been rising about the accumulation of hormones and other endocrine disrupting compounds in ground water sources. Environmental exposure to endogenous estrogen, 17β- estradiol (E_2), has been shown to affect the behavior, development, body proportions, and onset of ossification of *X. laevis* tadpoles, and the endocrine disruptor, 17α-ethynyl-estradiol (EE_2), a synthetic estrogen found in hormone replacement therapies and hormone-based birth controls, is a known water contaminant. In this study, *X. laevis* tadpoles were chronically exposed to EE_2 at two different concentrations, 2.5 nM and 5.0 nM, for 90 days. Animals exposed to EE_2 exhibited a decreased startle response compared to control animals. EE_2 exposed frogs also had an immature GI tract with more coils present compared to controls. Exposure to EE_2 also resulted in an overall proportional increase in body size. The concentration of EE_2 in ground water has been found as high as 2.3 µM, a 100-fold greater concentration than what was used to induce the behavioral and anatomical changes observed in the *X. laevis* tadpoles in this study.

Keywords: 17α-ethynylestradiol, *Xenopus laevis*, tadpole, development

Abbreviations: E_2, 17β- estradiol; EE_2, 17α-ethynylestradiol; GI, gastrointestinal; EDCs, endocrine disrupting compounds; BPA, bisphenol A; AR, Amphibian Ringer's; EtOH, ethanol; NF, Nieuwkoop and Faber; SVL, snout-vent length

1.0 Introduction

The accumulation of industrial waste and pharmaceuticals in ground water has been a recent rising concern world-wide [1]. Many compounds, both naturally occurring and synthetic, are not targeted for removal from wastewater, and these compounds can enter aquatic environments through discharge of liquid effluents from wastewater treatment facilities [1–5]. The improper disposal of industrial waste has the potential to affect the development and physiology of organisms living in freshwater environments [2].

Some contaminants from natural or industrial origins may act as endocrine disrupting compounds (EDCs), which can alter the activity of endocrine systems of organisms living in such waters [6–8]. Amphibians that live in freshwater ecosystems are at high risk for developmental defects because gases, ions, and lipid soluble compounds can easily permeate the outer membrane of their skin and may result in alterations of endogenous hormones [7,9].

Some recently investigated EDCs that have been found in ground water sources include cadmium, atrazine, bisphenol A (BPA), and estrogens [5, 6, 10, 11] Wastewater runoff from residential areas can contain 17α-ethynylestradiol (EE_2), the synthetic estrogen found in oral contraceptives and hormone replacement therapies. EE_2 is not currently targeted for removal from wastewater via water treatment plants and has been found in local surface waters at a concentration as high as 2.3 μM [4, 7].

There have been several studies examining the effects of EE_2 on different aspects of anuran development. Studies have shown that EE_2 exposure, both chronic and acute, can either delay larval development and metamorphosis in frogs or prevent full metamorphosis and development to adulthood [5, 6, 7, 12]. A one-time exposure of EE_2 to *Rana (Lithobates) pipiens* before metamorphosis resulted in a significant delay in the time taken to complete metamorphosis compared to controls [7]. These tadpoles were also significantly heavier and had a significantly longer snout-vent length than controls at the same age. Hogan et al. [7] also found reproductive abnormalities in those exposed to EE_2 compared to controls.

Although many studies explore the effects of EDCs on anurans, these studies lack information regarding the effects of EE_2 on other physiological systems, including the digestive and nervous systems. This study examines the effects of 90-day chronic EE_2 exposure on startle response, metamorphosis of the GI tract, and bone development and ossification of *Xenopus laevis*.

2.0 Materials & Methods

2.1 Animals and Husbandry

All animal husbandry and experimental protocols were approved by the Framingham State IACUC. About 100 *Xenopus laevis* tadpoles, Nieuwkoop and Faber Stages 40-47 [13], were purchased from NASCO (Fort Atkinson, WI). Tadpoles were randomly distributed across nine tanks, each containing 10 liters (L) of 1X Amphibian Ringer's solution (AR).

Tadpoles were fed Nasco Frog Brittle three times a week. Each tank was cleaned out three times a week at least three hours post-feeding by removing detritus and old food from the tank. A total of 2 L of AR was removed from each tank on cleaning days, which was immediately replaced with 2 L of fresh 1X AR solution.

2.2 Treatment

Of the nine tanks, three tanks contained an ethanol (EtOH) vehicle control, three tanks contained 2.5nM 17α-Ethynyl-estradiol (EE_2) and three tanks contained 5.0

nM EE_2. Stock EE_2 (Sigma; St. Louis, MO) was dissolved in an EtOH vehicle and mixed with 1X AR solution to make two concentrations of EE_2: 2.5 nM and 5.0 nM. When the treatment was added into the tanks, it was initially mixed with 200 mL of AR from the tank, and then this solution was added to the remaining AR in the tank to prevent pooling of the EE_2 solution.

2.3 Startle response

Once a week for twelve weeks, animals were subjected to a startle response test. The test was performed prior to feeding and cleaning of the tank to reduce the possibility of uncertainty in results due to stress of cleaning. Each tank was tapped in the same location on the outside of the tank each time (per Fraker and Smith, [9]). Movement of the tadpoles in response to the tap was recorded using a video camera. Videos were reviewed to determine the percentage of tadpoles in that tank that responded to the startle stimulus. A startle response was recorded if an animal exhibited any sharp or darting movement immediately after the stimulus was applied to the tank [9]. The test was only run once a week to avoid desensitization to the stimulus.

2.4 Developmental Staging and External Measurements

After 90 days of treatment, all remaining animals were sacrificed by immersion in 10% benzocaine followed by fixation in 10% formalin. All specimens were staged following Nieuwkoop and Faber [13] (abbreviated throughout as NF) and stored at 4^oC. Specimens verified to be at Stage NF 66, or having completed metamorphosis, were selected and set apart for external and internal analysis. Stage 66 specimens were then placed under an Olympus 8ZN10 Infinity 1 dissecting microscope connected to a computer and visualized using Infinity Capture Software. Snout-vent lengths (SVL) were measured using electronic Vernier calipers.

2.5 Internal Observation/Organ Removal

Each specimen at Stage 66 was individually placed in a glass specimen dish containing spring water and placed under an Olympus 8ZX10 Infinity 1 microscope. Specimens were cut medially on the ventral side with dissection scissors and opened so that the internal organs in the central cavity could be visualized. Each specimen was then photographed undisturbed using Infinity Capture software. The GI tract (esophagus to rectum) was carefully removed using forceps and placed into another glass dish containing spring water. The necropsied animal was placed back into its original container for further analysis. Each GI tract was analyzed by counting the number of coils, as defined as at least a 90^o change in direction present in the tract. The GI tract was then photographed and placed in a unique vial containing formalin.

2.6 Clearing and Staining Procedure

Specimens were cleared and double stained with Alcian Blue and Alizarin red to visualize cartilage and bone. Methods were modified from Taylor and van Dyke [14]. Twenty-compartment plastic tackle boxes with drilled holes in each compartment were used in the clearing and staining procedure. Each organism was placed in a unique compartment and labeled. Each box was set in a 9x13 glass Pyrex baking dish used to hold the various solutions in the procedure. Each solution was poured into the Pyrex dish, followed by immersion of the tackle box of organisms. Organisms were first subjected to two one-hour-long washes in distilled water after removal from formalin. Organisms were then placed in a 1.5 X solution of Alcian blue solution for two hours to stain cartilage. The 1.5 X Alcian

blue solution was made by dissolving 4.50 mg Alcian blue 8Gx in 1050 mL 95% EtOH and 450 mL glacial acetic acid. After immersion in Alcian blue, organisms were then sequentially placed in 70% EtOH, 50% EtOH, 30% EtOH and then 100% d-H2O for 45 minutes each. Next, organisms were placed in 30% saturated sodium borate for 10 minutes and then placed in 10% pancreatin overnight to digest body tissues.

After removal from pancreatin, organisms were rinsed in three 5-minute washes of 0.5% potassium hydroxide (KOH). Organisms were then placed in an Alizarin red solution, to visualize bones, overnight. Alizarin red was made by dissolving 0.1 g of Alizarin red powder in 1 L of 0.5% KOH. After removal from Alizarin red, organisms were rinsed in two 5-minute washes of 0.5% KOH. Organisms were then transferred through a 0.5% KOH/glycerin series (3:1, 1:1 and 1:3) for two days per step. Then 10 mL of 3.1% hydrogen peroxide was added per 1 L of solution in the Pyrex dishes to bleach out the pigments of the skin. Dishes were placed in windows to accelerate the bleaching. Solutions were changed every day regardless of the solution composition. After transferring through the series, organisms were placed into individual labeled vials containing 100% glycerin.

2.7 Bone Visualization and Measurements

An Olympus 8ZN10 Infinity 1 dissecting microscope and digital Vernier calipers were used to measure the individual bones of the forelimbs and hind limbs. An average measurement was calculated by measuring the left and right humerus and radioulna bones of the forearm and the left and right femur, tibiofibula, and tibiale bones of the hind limb. Averages were calculated per treatment, per bone.

2.8 Statistical Analysis

For each treatment group, percentages resulting from startle response data were averaged for the entire 12 week run of tests. Averages were compared by a one-way analysis of variance (ANOVA) and post-hoc Tukey's test. SVLs were averaged by treatment and compared by a one-way ANOVA and post-hoc Tukey's test. To normalize for body size, the number of GI coils and individual bone lengths were analyzed as a proportion of SVL. Averages were calculated for coils/mm SVL and mm bone/ mm SVL and analyzed via one-way ANOVA and post-hoc Tukeys. All statistics were performed using VassarStats.

3.0 Results

3.1 Startle Response

Animals treated with either 2.5 or 5.0 nM EE_2 exhibited a significantly lower startle response compared to controls ($p<0.05$, Figure 1). The startle response of both the 2.5 and 5.0 EE2-treated groups was only about two-thirds of the response in control group.

3.2 Snout-Vent Length (SVL):

Average SVL was calculated for each of the three treatment groups for frogs that had completed metamorphosis (NF66), and compared for significance (Figure 2). The average SVL of organisms raised in the control environment was significantly shorter than those raised in 5.0 nM EE_2, ($p<0.05$) but not significantly different from 2.5 nM EE_2 treatment group.

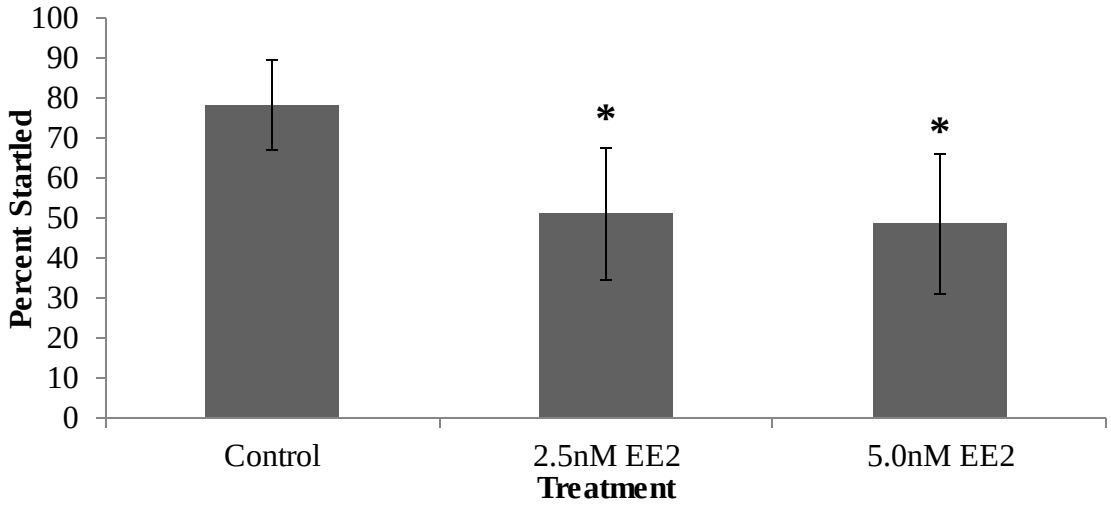

Figure 1: The percentage of tadpoles exhibiting a startle behavior in response to a tap on the side of the tank was calculated over 12 weeks. Tests were conducted once a week. Exposure to a both 2.5 nM and 5.0 nM EE_2 resulted in significantly fewer number tadpoles exhibiting a startle response (Tukey's, $p<0.05$). Error bars represent standard deviation.

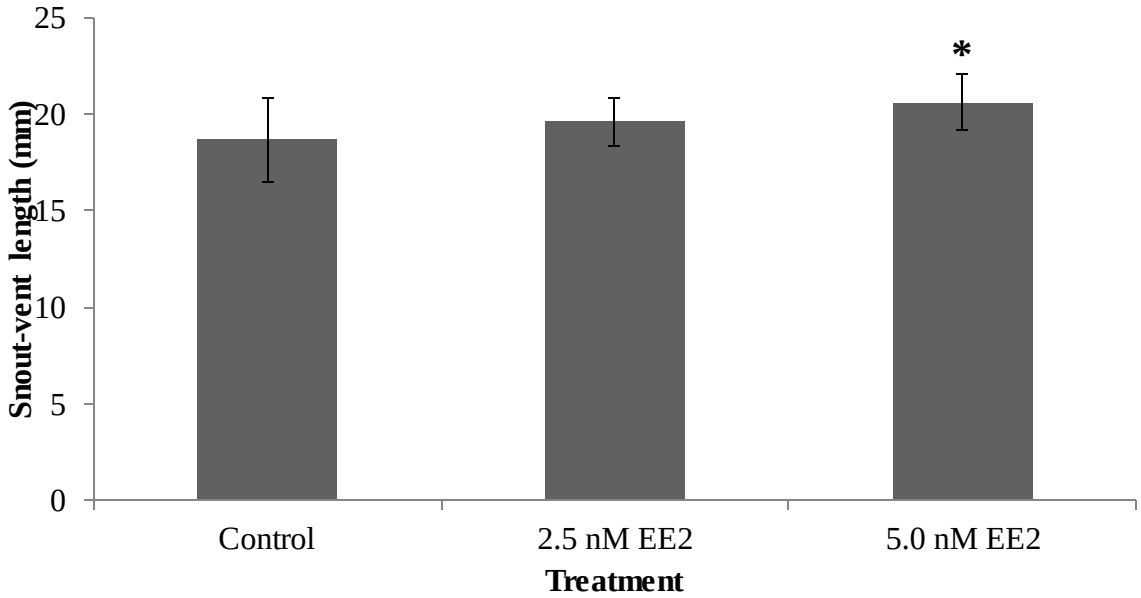

Figure 2: Animals treated with 5.0nM EE_2 had a larger SVL at Stage 66, compared to control specimens (Tukey's, $p<0.05$). Error bars represent standard deviation.

3.3 Number of Coils present in Gastro-intestinal Tract:

The ratio of the number of coils in the GI tract to SVL was calculated for each treatment group at NF66 to take body size into account (Figure 3). There was a significantly greater number of coils per millimeter of body length present in both the 2.5nM and 5.0 nM EE_2 treated organisms compared to control ($p<0.01$).

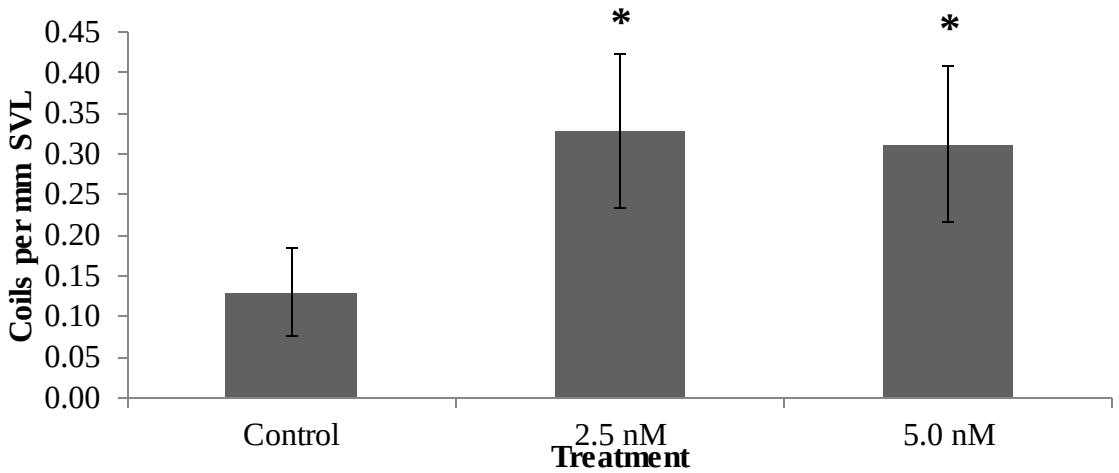

Figure 3: Average ratio of number of GI coils to SVL of Stage 66 *X. laevis* after chronic exposure to different concentrations of EE_2. Control animals contained significantly fewer coils, when compared to 2.5 nM and 5.0 nM EE_2 treated animals (Tukey's, $p<0.01$). Error bars represent standard deviations.

3.4 Bone Length

To take body size into account, individual bone lengths were normalized for body size and data were calculated as a proportion of individual bone lengths to SVL for frogs at NF66 (Figure 4). For each of the five bones measured: humerus, radioulna, femur, tibiofibula and tibiale, there was no significant difference between treatments ($p=0.12$ for humerus, $p=0.24$ for radioulna, $p=0.85$ for femur, $p=0.50$ for tibiofibula, and $p=0.27$ for tibiale).

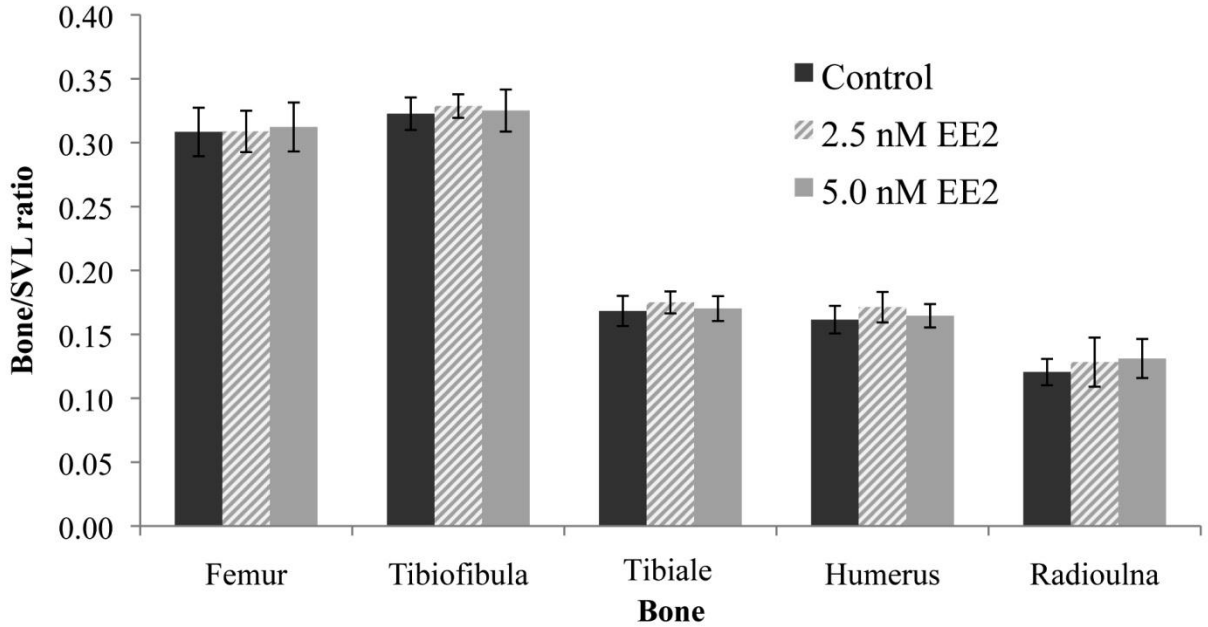

Figure 4: Average ratio of bone length to SVL of Stage 66 *X. laevis* after chronic exposure to different concentrations of EE_2. There were no significant differences in the proportions of any bone examined (ANOVA, $p> 0.05$). Error bars represent standard deviation.

4.0 Discussion

The African clawed frog, *X. laevis*, has been used extensively to examine the effects of many hormones on different developmental processes. *X. laevis* spends its entire life cycle in fresh water environments, including the period of metamorphosis [7, 15]. Because of this, *X. laevis*, especially during the larval stage, is vulnerable to foreign compounds in the water including gases and lipid soluble compounds such as steroid hormones. Lipid soluble steroid hormones can easily permeate through the thin skin of *X. laevis* and be incorporated into the tissues with the potential to alter behavior and developmental processes [16].

Estrogens have been found to alter the activity of the vertebrate nervous system [17], the responses to stress [18] and the development and maintenance of the skeleton [19]. Estradiol is considered an anxiolytic compound, and can decrease the synthesis of stress hormones in female rats and zebra fish [17,18]. Imbalances in estrogen levels may affect the survival of organisms by decreasing their responses to stress and putting the organisms at a higher risk for predation [20]. Also, embryos developed in water containing E_2 suffered suppressed organogenesis of the nervous system [21]. In the present study, EE_2 treatment at either concentration (2.5 nM and 5.0 nM) decreased the response *X. laevis* exhibited towards the basic stimulus of a startle, suggesting that EE2 is anxiolytic in the anuran nervous system.

There is a dramatic remodeling and restructuring of the *X. laevis* GI tract during metamorphosis. An immature tadpole GI tract is a long tube with a single layer of epithelial cells that coils up and around itself [22–24]. As tadpoles, *X. laevis* are herbivores. Evolutionarily, herbivorous animals have evolved to have longer GI tracts. A longer GI tract is more efficient for plant digestion [25]. During metamorphosis, a *X. laevis* tadpole transitions in diet from an herbivorous animal to an omnivorous animal. To accommodate the change in diet, the single-layered tube congregates into a thicker multi-layered tube with crypts and villi [23, 24]. By the end of metamorphosis, the epithelial cells of the tadpole GI tract are congregated into a thick multi-cellular lining that fold into multiple ridges and intestinal folds which condenses the GI tract to about 25% its original length [22, 24]. At this point, the GI is no longer coiled and presents as a linear tube. This structure is adequate for an omnivorous organism [25]. Previous studies have found that exposure to estradiol (E_2) or the weak estrogen mimic nonylphenol polyethoxylate during metamorphosis resulted in abnormal coiling of the tadpole GI tract [16, 26]. Embryos developed in water containing E_2 suffered suppressed organogenesis of digestive organs, abdominal swelling, and retardation of gut development [21]. In this study, treatment at either concentration significantly increased the number of coils present in the GI tract. Those exposed to EE_{-2} had a significantly higher number of coils and ratio of coils to body length suggesting that treated frogs had an immature form of the GI tract.

The development of an ossified skeleton is another process that occurs during metamorphosis. Bauer-Dantoin and Meinhardt [19] found that exposure of *Xenopus laevis* to estradiol (E_2), resulted in an accelerated skeletal development and onset of ossification in limb bones. Estrogen was also found to accelerate ossification and epiphyseal fusion in rabbits. Rabbits exposed to estrogen in two different experiments were found in both to have accelerated epiphyseal fusion, decreased chondrocyte levels and chondrocyte proliferation, shorter hind limbs (femur and

tibia), and shorter growth plates compared to controls [27, 28].

In the present study, EE_2 treatment at 5.0 nM significantly increased the snout-vent length and increased size of the two forelimb bones and the tibiofibula and tibiale; however, treatment with EE_2 did not have an effect on body proportions when bone length was normalized for body size. Tadpoles exposed to EE_2 during metamorphosis were significantly longer as adults when compared to controls. However, for each of the 5 bones measured, there was no significant effect of treatment on the ratio between bone lengths to SVL ratio, suggesting that estrogen treatment resulted in proportionately larger frogs. Ossification may have started earlier in our samples; however, it appeared to have no detriment on the frogs. It is important to note that any early influences of EE_2 on ossification may have been missed due to examination at Stage NF 66 rather than earlier in the developmental process.

Currently, there are over 6400 known anuran species [29] and the effects of estrogenic compounds may differ among them. *Rana (Lithobates) pipiens* exposed to estrogenic compounds were not significantly smaller or larger compared to controls [30]. In contrast, the average SVL of *Rana (Lithobates) sylvatica* tadpoles exposed to EE_2 was significantly larger than that of controls [30, 31]. Alternatively, the variable results from different studies could be attributed to the timing of the exposure and the availability of estrogen to receptors, the concentration of the estrogenic compound, or the organism's stage of development. This study adds to the conversation that estrogenic compounds are affecting aquatic organisms; however, how, on a cellular level, estrogens are affecting these organisms is still unknown.

Overall, this study shows that treatment with EE_2 has affected both the startle response and the developmental processes underlying metamorphosis. These results imply that organisms living in fresh water environments may be subject to alterations in their behaviors and metamorphosis due to wastewater contaminants. EE_2 is only one of many EDCs present in wastewater that are not subject to removal from wastewater sources. This study shows that significant developmental changes occur in *X. laevis* when tadpoles are treated with small concentrations of EE_2 (2.5 nM and 5.0 nM) for 90 days after hatching. Concentrations of EE_2 in ground water sources have been found as high as 2.3 μM [4], a 100-fold greater concentration than used in this study. Further research needs to continue in this area to determine the full impact that these EDCs are having on anuran populations.

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