

# Morphometric and phenotypic effects of 17 $\alpha$ -ethynylestradiol exposure in the wood frog (*Rana sylvatica*)

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## Abstract

Estrogenic chemicals in the environment have been implicated in causing a variety of adverse effects in exposed organisms. Exposure to estrogens, especially at critical times during sexual development, can cause feminization and/or demasculinization of males of many amphibian species. 17 $\alpha$ -ethynylestradiol (EE2), a hormone analog commonly used in oral contraceptives, is an estrogenic chemical of particular environmental concern due to its great potency and ubiquitous release in sewage treatment plant effluents. In the current experiment, EE2 was used as a model compound in an exposure with wood frog (*Rana sylvatica*) larvae to attempt to discover links between biologically relevant effects and molecular events. The wood frog is native to temperate areas in North America and common throughout much of its large range. Wood frogs are sensitive to EE2 exposure during sexual differentiation and undergo male-to-female sex reversal when exposed at adequate concentrations. To cover the full spectrum of sex reversal, wood frog tadpoles were exposed to 1, 10, or 100  $\mu$ g/L EE2 (nominal). Tadpoles were sampled at Gosner Stages 26, 30/31, 33/34, and 35/36 to determine the effects of EE2 on tadpoles that are undergoing sexual differentiation. The remaining tadpoles were grown until metamorphic climax or 108 d. Total mortality over the course of the experiment ranged from 3.4-11%. There were no significant treatment related differences in tadpole weight or snout-vent-length (SVL) at any time during the experiment. In addition, there was no difference in time to metamorphosis between treatments. Sex ratios were significantly female-biased in all EE2 treatments. Male:female ratios were 1:1.4, 1:4.6, and 1:8.1 in the solvent control (SC), 1 and 10  $\mu$ g/L EE2 treatments, respectively. 100% of individuals in the 100  $\mu$ g/L EE2 treatment developed as phenotypic females. In short, these results indicate that treatment with EE2 caused male-to-female phenotypic sex reversal without impacting the other biological parameters measured in the current study. Histological analysis of gonads to verify phenotypic sex ratios and assign phenotypes to ambiguous individuals is ongoing. Transcriptome analysis of tadpole RNA samples using 'sequence by synthesis' technology will begin in late 2010. Comparison of the transcriptomes of exposed and unexposed individuals should allow determination of some of the molecular events underlying the biologically relevant phenomenon of sex reversal.

## Introduction

- Exposure to potent estrogens, including 17 $\alpha$ -ethynylestradiol (EE2), can interfere with sexual differentiation in some frog species
- Sex reversal or altered development is possible during the aquatic larval period
- Thus, frogs/tadpoles could be impacted by estrogenic compounds present in the aquatic environment
- Disruption of sexual development, including sex reversal, is considered a biologically relevant phenomenon
- The wood frog (*Rana sylvatica*) is a common amphibian native to large areas of temperate North America
- Wood frogs range further north than most other amphibian species and are an integral part of many northern ecosystems
- Wood frogs are sensitive to EE2 exposure and will undergo male-to-female sex reversal at adequate doses
- The wood frog can be used as a model species in studies of estrogen-induced sex reversal
- Other biologically relevant endpoints, such as size at and time to metamorphic climax, can be evaluated in amphibian-based studies
- EE2 and the wood frog were used as a model chemical and species, respectively, in a study designed to determine the molecular events that orchestrate the biologically relevant event of phenotypic sex reversal
- 'Sequence by synthesis' technology will be used to determine changes in gene expression that accompany sex reversal
- This technology allows rapid sequencing of the entire transcriptome of an organism
- Transcriptomes of exposed and unexposed individuals can then be compared to determine the changes in gene expression responsible for sex reversal

## Experimental Design & Methods

### II. Experimental Design & Maintenance

- Treatments
  - Control and 0.0025% ethanol solvent control (SC)
  - 1, 10 and 100  $\mu$ g/L EE2
- Exposure Scenario
  - 50% static water renewal every 24 h
  - Three replicate tanks per treatment group
  - 50 tadpoles per tank at experiment initiation
  - Tadpoles sampled at 4 time points in sexual development
    - Gosner Stages 26, 30/31, 33/34, and 35/36
    - Days 14, 25, 35, and 54 of exposure
    - 15 tadpoles grown to metamorphic climax or 108 d, whichever occurred first
- Experiment maintenance
  - Tadpoles fed *ad libitum* once daily
  - Temperature maintained at 18-22°C
  - DO, pH, conductivity and temperature monitored daily
  - EE2, ammonia, nitrate and nitrite concentrations monitored weekly

**Figure 1:** Collection of wood frog egg masses  
Six egg masses were collected from a pond in the Saskatoon area on April 8, 2010. Three of the masses at their *in situ* communal deposition site (L) and a close-up of one egg mass (R) are shown.

### I. Egg Mass Collection & Laboratory Acclimation

- 6 egg masses (Fig. 1) were collected from a pond near Saskatoon on April 8, 2010 (SK Special Permit #10F0U059)
- Egg masses were immediately transferred to the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan, and acclimated to laboratory conditions and water
- Eggs began hatching on April 12, 2010 and most larvae were free-swimming on April 15, 2010
- EE2 exposure was initiated on April 15, 2010

**Figure 2:** Exposure Setup  
Experimental area in the ATRF



### III. Experiment Termination

- Tadpole Time Point Subsamples
  - Gosner stage, weight and snout-vent-length (SVL) determined
  - Flash frozen whole for molecular analysis
- Tadpole Grow-Out
  - Tadpoles that reached metamorphic climax (Gosner Stage 42+)
    - Sacrificed at metamorphic climax
    - Days to metamorphosis (DTM) determined
    - Gosner stage, weight, and SVL determined
    - Brain and liver flash frozen for molecular analysis
  - Gross phenotypic sex determined with dissecting microscope
  - Body, including gonads, preserved in 10% formalin for determination of histological phenotypic sex
  - Tadpoles that failed to reach metamorphic climax
    - Sacrificed at 108 d (July 24, 2010)
    - Samples collected as for tadpoles that reached metamorphic climax

### IV. Statistics

- Survival analysis (Systat 12) was used to determine average DTM
  - Allowed inclusion of individuals that failed to reach metamorphic climax
- Weight, SVL, and DTM data were analyzed with ANOVA tests (Systat 12)
- Sex ratio data were analyzed with Fisher's Exact Tests (SPSS)
- Due to differences between SC and control, SC is used for all comparisons
- Due to high mortality (50%) in one triplicate tank, only 2 tanks from the 100  $\mu$ g/L EE2 treatment were used in all analyses

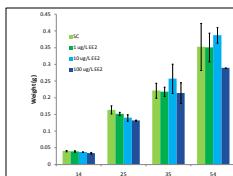
## Results

### I. Total Mortality

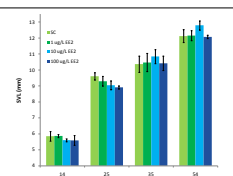
- Average mortality for all treatments was 7.7±1.5%
- Mortality ranged from 3.4-10.5%
- SC had the least mortality (3.4%)
- Mortality in the EE2 treatments ranged from 7.4-10.1%
- There were no statistically significant differences between treatments

### II. Weight and length during development

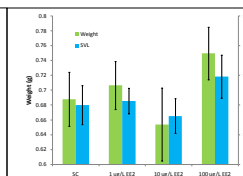
- Tadpoles grew at the same rate in all treatments
- Weight (Fig. 3) and SVL (Fig. 4) increased over time
- Within each time point, there were no significant differences in weight or SVL
- At metamorphic climax, all tadpoles were similar in size (Fig. 5)
- Average weight ranged from 0.65-0.75 g
- Average SVL ranged from 16.8-17.5 mm



**Figure 3:** Tadpole Weight during Larval Development  
Weight per tadpole (Mean±SD) in each treatment is shown for 14, 25, 35 & 54 d. Within time points, there were no significant differences between treatments.



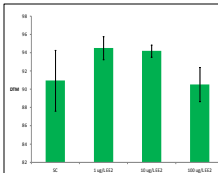
**Figure 4:** Tadpole SVL during Larval Development  
SVL per tadpole (Mean±SD) in each treatment is shown for 14, 25, 35 & 54 d. Within time points, there were no significant differences between treatments.



**Figure 5:** Weight and SVL at Metamorphic Climax  
Weight and SVL per tadpole (Mean±SD) at metamorphic climax is shown. There were no significant differences between treatments in weight or SVL.

### III. Days to Metamorphic Climax (DTM)

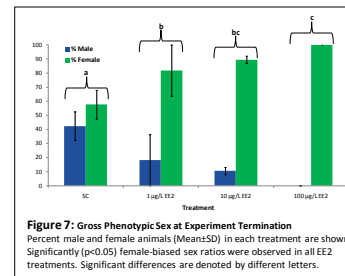
- On average, tadpoles took 92.5±1.1 d to reach metamorphic climax (Gosner Stage 42+)
- Average DTM ranged from 90.5-94.5 d
- EE2 treatment did not significantly impact DTM (Fig. 6)



**Figure 6:** Average Days to Metamorphic Climax  
Average time to metamorphic climax (DTM) is shown (Mean±SD). There were no significant differences between treatments.

### V. Phenotypic Sex Ratios: Gross Morphology

- Some individuals could not be assigned a definite phenotype and were classified as ambiguous
- Some ambiguous animals were probably intersex, but most were simply too small to assign a phenotype
- Histological analysis is needed to assign these animals a phenotype
- The number of animals of ambiguous phenotype did not differ across treatments
- Thus, the ambiguous animals were removed from the sex ratio analysis
- Sex ratio analysis
  - SC sex ratio did not differ significantly from the expected 1:1 ratio
  - All male:female sex ratios in the EE2 treatments were significantly more female-biased than the SC sex ratio (Fig. 7)
    - SC = 1:1.4 (42% male, 58% female)
    - 1  $\mu$ g/L EE2 = 1:4.6 (18% male, 82% female)
    - 10  $\mu$ g/L EE2 = 1:8.1 (11% male, 89% female)
    - 100  $\mu$ g/L EE2 = 0:1 (0% male, 100% female)
  - Observed sex ratios are most likely due to male-to-female phenotypic sex reversal as a result of exposure to EE2 during sexual differentiation



**Figure 7:** Gross Phenotypic Sex at Experiment Termination  
Percent male and female animals (Mean±SD) in each treatment are shown. Significantly (<0.05) female-biased sex ratios were observed in all EE2 treatments. Significant differences are denoted by different letters.

## Discussion & Ongoing Research

### I. Discussion

- Multiple biological endpoints were examined, including weight and SVL at various time points, time to metamorphosis, and sex ratios
- As expected for this model exposure, only sex ratios were impacted by exposure to EE2 at the concentrations tested
  - Skewed sex ratios are most likely due to male-to-female phenotypic sex reversal of genetic males exposed to EE2 during sexual differentiation
  - Currently, there is no way to verify which individuals underwent sex reversal since the wood frog has no known sex-linked gene
- Sex reversal is a biologically relevant event
- Samples from the current experiment can now be examined with molecular techniques to determine the molecular events involved in the sex reversal process
- Since the wood frog genome/transcriptome is poorly characterized, open format techniques such as *illumina* or 454 transcriptome sequencing may be more informative than traditional technologies

### II. Ongoing Research

- Histological analysis of the gonads to confirm or assign phenotypic sex to individuals from the experiment
- Molecular analyses
  - Determination of a marker(s) of genetic sex in the wood frog
  - 'Sequence by synthesis' transcriptome analysis to determine differences in gene expression between EE2 exposed and unexposed individuals
  - RT-PCR verification of genes that are differentially expressed in EE2 exposed and unexposed individuals

## Acknowledgements

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