17β-estradiol accelerates the rate of skeletal calcium incorporation during early development in *Danio rerio*

Jimmy Rager, Cailyn Scanlan, Matt Slitzky, Angela Bauer and V. McNeil Coffield

Department of Biology, High Point University

Abstract

Estrogen is known to play a significant role in bone development and ossification in mammals. To date, however, little is known about the sex hormone's role in bone ossification in the zebrafish model, *Danio rerio*. In this study, zebrafish embryos were raised in various concentrations of 17β-estradiol (10^{-11} M - 10^{-9} M) for 14 days. Two staining methods, Alcian Blue/Alizarin Red and calcein were used to determine bone ossification and development. First, embryos treated with 17β-estradiol were stained with Alcian Blue and Alizarin Red, which stain for cartilage and bone, respectively. The specimens were scored for the extent to which their embryonic skeleton had ossified and the length of various endochondral bones and total body length were measured. The second method of staining used calcein dye, which adheres to calcium minerals and fluoresces under GFP-similar-wavelengths, indicating the onset of bone ossification. Lateral/dorsal images of the embryos were obtained from day six through day ten and each of the first fifteen vertebrae were then blindly scored based on the amount of stain present (0= none, 1= x<30%, 2= 30%<x<70%, 3= x>70%). Preliminary data from the first staining method suggests a significant dose-dependent increase in total body length and also an increase in the length of specific endochondral bones compared to untreated controls ($p<0.05$ and <0.005, respectively). Calcein staining data supports the notion that 17β-estradiol increases the rate of vertebral bone ossification, most drastically at day 7. Our data supports the hypothesis that 17β-estradiol influences the rate of calcium incorporation during early development and offers a potential causative agent for the skeletal abnormalities observed in animals living in areas contaminated with high levels of endocrine disruptors.

Introduction

17β-estradiol, also known as estrogen (E2) is a well known female sex hormone that is synthesized from cholesterol and plays a role in a variety of physiological processes, including brain function, secondary sex characteristics and bone development. E2 is a small non-polar molecule can easily pass through the cell membrane. It binds with high affinity to estrogen receptor alpha/beta (ERα/β) in the nucleus or cytoplasm (Fig 1). This interaction results in a conformational change and forms a homodimer complex that translocates to specific genomic regions. This complex binds to CG rich Estrogen Responsive Elements (EREs) in the promoter regions of many genes and regulates production of cell proliferation genes (E2F, Sp1), survival genes (Bcl2) and Bone Matrix Proteins (TGF-β). The ability to regulate these proteins involved with bone development had led to the hypothesis that increased estrogen exposure can result in deviated bone growth compared to controls.

Previous studies have demonstrated a strong correlation between the rate of skeletal abnormalities found in aquatic species and the presence of fertilizers containing various estrogenic compounds. This study aims to identify a link between estrogen activation and fish bone development. Future studies will examine the affects of additional estrogenic and anti-estrogenic compounds and their potential role in the regulation of skeletal development.

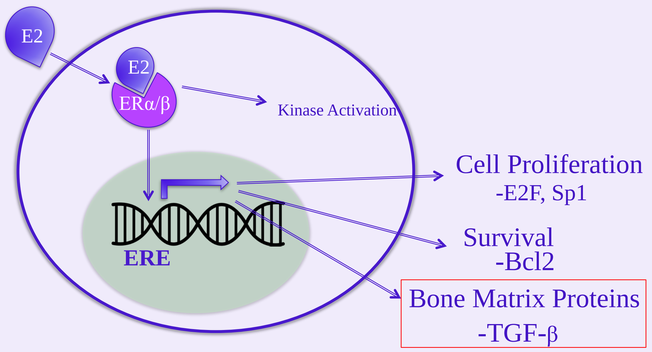
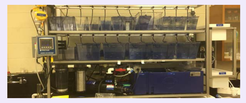


Figure 1. Basic 17β-estradiol Mechanism of Action

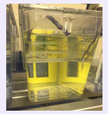
Materials and Methods



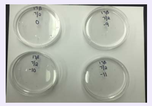
Full tank system

Breeding chambers established

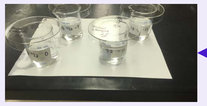
-1 male
-2 females



Embryo Harvest and Treatment



Day 5



Embryo Maintenance

-Solutions changed daily
-Fed paramecium at Day 5
-Incubated at 28.5 ˚C

Fixation and Staining

Alcian Blue/Alizarin Red

-Stain for cartilage and bone, respectively

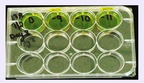
Leica 205 Stereoscope

+

Measurements of total body length and specific endochondral bones were taken

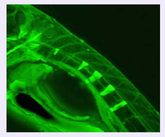
Calcein

- adheres to calcium minerals and fluoresces under GFP-similar wavelengths



Zeiss Spinning Disk Confocal Microscope

Z-Stack and Photoshop Compilation



Scoring System

Bone Ossification Index

DAY 6		1	2	3	4	5	6	7	SUMMATION
	CONTROL	3.00	0.00	0.00	1.67	1.50	0.67	0.50	7.35
	-9	3.00	0.00	0.00	1.67	2.67	1.50	0.00	8.83
	-10	3.00	0.00	0.00	1.83	3.00	0.83	0.00	8.67
	-11	3.00	0.00	0.00	1.00	2.67	1.00	1.00	10.67

Data Analysis

Alizarin Red and Alcian Blue Results

Table 1. Effects of 17β-estradiol on total body length

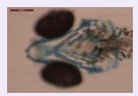
Control

10^{-10} M

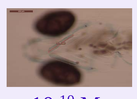
10^{-9} M

	Average Length (mm)	% Change	p-value
Control	4.014	N/A	N/A
10^{-10} M	4.1033	2.20%	0.291
10^{-9} M	4.2345	5.50%	0.0148 ***

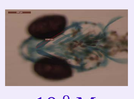
Table 2. Effects of 17β-estradiol on ceratohyal bone length



Control



10^{-10} M



10^{-9} M

	Average Length (mm)	% Change	p-value
Control	0.248	N/A	N/A
10^{-10} M	0.268	8.00%	0.00214 ***
10^{-9} M	0.290	16.90%	0.0000465 ***

Calcein Results

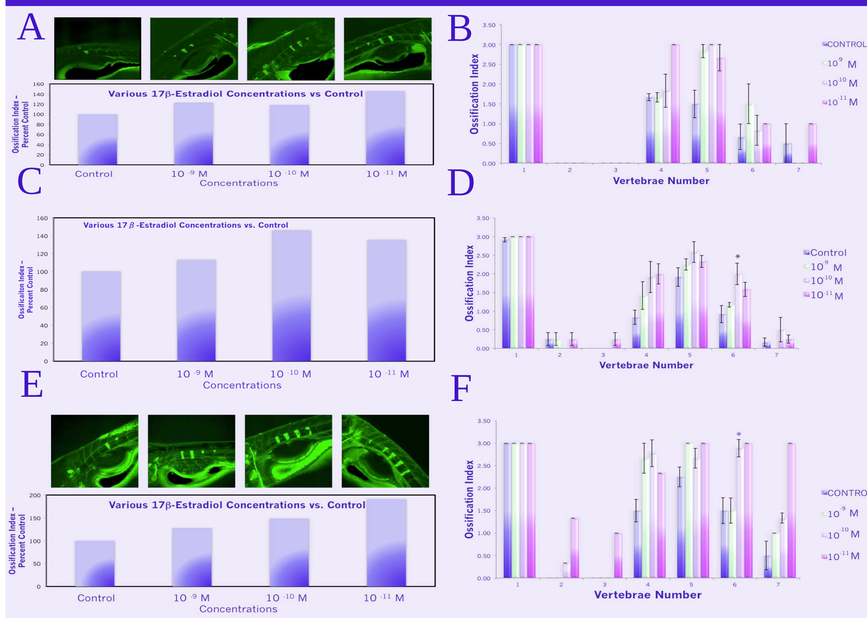


Figure 2. Calcein staining of embryos at various concentrations at days 6, 7, and 8 days post fertilization (dpf). Figures 2A, 2C, and 2E represent the Total Bone Ossificaiton Index (BOE) for all vertebrae. Figures 2B, 2D, and 2F have been broken down at each individual vertebrae. 2A and 2B – 6dpf. 2C and 32 – 7 dpf. 2E and 2F – 8 dpf.

Conclusions

- 17β-estradiol impacts ossification and bone development in *Danio rerio*
- Lower-doses of 17β -estradiol accelerates the rate at which the vertebrae ossify
- Higher-doses of 17β -estradiol increases total body length and length of ceratohyal bones
- Based on results observed previously by Dr. Bauer, these concentrations are analogous to those observed in the environment and their impact and mechanisms should be further evaluated

References

•Bauer-Dantoin, A.C and Meinhardt, D. J. (2010), 17b-Estradiol Exposure Accelerates Skeletal Development in Xenopus laevis Tadpoles. The Anatomical Record 293:1880-1886.

•Kimmel, C.B, Miller, C.T., and Moens, C. B. (2002), Specification and Morphogenesis of the Zebrafish Larval Head Skeleton. Devlopmental Biology 233(2): 239-257, doi:10.1006/dbio.2001.0201.

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