Title
Differential Estrogen Sensitivity Among Populations of African Clawed Frogs

Author
Luong, Xuan Giang

Publication Date
2016

Peer reviewed|Thesis/dissertation

Differential Estrogen Sensitivity Among Populations of African Clawed Frogs

by

Xuan Giang Luong

A dissertation submitted in partial satisfaction

of the requirements for the degree of

Doctor of Philosophy

in

Integrative Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Tyrone B. Hayes, Chair
Professor George E. Bentley
Professor Lance J. Kriegsfeld

Fall 2016

Abstract

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Xuan G. Luong

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Estrogens are a class of steroid hormones ubiquitous and crucial to organismal reproductive success throughout the vertebrate phylum. It is physiologically important to both males and females, playing a crucial role in gonad development, spermatogenesis, oogenesis, bone development, and sex behavior. Localized overproduction of the estrogen is significant in the development and progression of hormone-dependent cancers (e.g. cancers of the breasts, endometrium, etc.). Many chemicals that increase the production of estrogen or mimic its action have contributed to the global decline of aquatic life due to adverse effects on species' development and reproductive health. Comparable responses to environmental contaminants have been observed in humans. It is evident that not all populations, individuals, or tissues are equally affected by hormone-driven ailments. I aim to elucidate the basis of population differences in estrogen susceptibility using the African clawed frog (*Xenopus laevis*). This knowledge will not only contribute to the field of developmental endocrinology, but may also hold great significance in environmental toxicology and animal conservation.

Chapter One reviews the role of estrogen in amphibian development, the impacts of endocrine disruptors on animal health, and the significance of acknowledging and characterizing response variation in animal models used in scientific studies.

Chapter Two describes a study where I examined how two source populations of *X. laevis* display different susceptibilities to the feminizing effects of 17-beta estradiol (E2). All-male, ZZ-tadpoles from a store-bought population, Xenopus Express (XE), and from a wild-caught population, San Diego (SD), were treated with 3.00 ng/ml E2 starting at 10 days post-hatch until metamorphosis. Animals were then sacrificed, gonad sex was determined via dissection, and sex ratios were calculated. Experiments were conducted with multiple parents within each population as well as with familial replicates in order to verify sex reversal trends. The data indicated that, within a species, there are inherent differences in gonad susceptibility to the feminizing effects of E2 and implied varying susceptibility to the feminizing effects of endocrine disruptors.

In Chapter Three, I examined whether differences in E2 susceptibility span tissue types and developmental stages. Adult, SD and XE males were treated with increasing concentrations of E2 for 12 hours. Expression of E2-responsive genes, *vtga2* and *esr1*, in the liver was quantified via qRT-PCR and compared between the two populations. XE and SD animals showed different fold-changes in *vtga2* expression in response to E2 treatment, and there were disparities between *vtga2* and *esr1* expression. These genes may be used as indicators of E2 exposure, but may not necessarily serve as effective tools to measure the degree of *in vivo* exposure.

Chapter Four discusses the results of an RNA-Seq experiment examining differential gene transcription between control and E2-treated SD and XE groups. All male, ZZ tadpoles were treated with 3.00 ng/ml E2 starting at 10 days post hatch (dph) and sacrificed when they reached NF stages 52-53 (during which sex differentiation occurs). The gonads were then dissected out, mRNA was extracted, and a cDNA library was constructed and sequenced. *ahr.S*, *cyp2c8.2.L*, *cyp19a1.L*, *dmrt1.S*, and *hsd17b7.S* were differentially expressed genes between the two populations that we recognized to have a role in sex differentiation and steroidogenesis. Based on the expression profiles, we proposed a mechanism underlying susceptibility to feminization, which involves both the suppression of masculinizing genes and the upregulation in feminizing genes.

This dissertation is dedicated to our lab animal friends.

"Altruism has always been one of biology's deep mysteries. Why should any animal, off on its own, specified and labeled by all sorts of signals as its individual self, choose to give up its life in aid of someone else?"
—Dr. Lewis Thomas, *Altruism*—

Table of Contents

page

Dedication ..... **i**

List of Figures ..... **iii**

List of Tables ..... **iv**

Acknowledgements ..... **v**

Chapter 1
The Importance of Estrogen in Amphibians **1**

Chapter 2
Characterization of Differential Estrogen Susceptibility in Populations of African Clawed Frogs **16**

Chapter 3
Differential Estrogen Susceptibility Spans Tissue Types and Developmental Stages **38**

Chapter 4
RNA-Seq Confirms Population Gene Expression Differences in the Developing Gonads **71**

Chapter 5
Conclusions **108**

List of Figures

page

Chapter 2

Figure 1A: Breed-wise ZZ-female percentages **32**
Figure 1B: Average ZZ-female percentages **33**
Figure 2A: Familial replicates: breed-wise ZZ-female percentages .. **34**
Figure 2B: Familial replicates: average ZZ-female percentages **35**

Chapter 3

Figure 1A: Sample-wise *vtga2* v. E2 treatment duration **54**
Figure 1B: Average fold change *vtga2 v.* E2 treatment duration **55**
Figure 2: Average E2 plasma concentration v. E2 treatment **56**
Figure 3A: Sample-wise *vtga2* v. E2 treatment **58**
Figure 3B: Average fold change in *vtga2* v. E2 treatment **59**
Figure 4A: Sample-wise *esr1* v. E2 treatment **61**
Figure 4B: Average fold change in *esr1* v. E2 treatment **62**
Figure 5: Sample-wise *vtga2* v. E2 plasma concentration **64**
Figure 6: Sample-wise *esr1* v. E2 plasma concentration **65**
Figure 7: Sample-wise *vtga2* v. sample-wise *esr1* **66**

Chapter 4

Figure 1: Multidimensional scaling (MDS) plot **91**
Figure 2: Gene-wise biological coefficient of variation (BCV) plot **92**
Figure 3: Steroid hormone biosynthesis in *Xenopus laevis* **106**
Figure 4: Mechanism of population-dependent E2 susceptibility .. **107**

List of Tables

page

Chapter 2
Table 1: Breed information **31**
Table 2: Estradiol-induced sex reversal literature review **36**

Chapter 3
Table 1: ANOVA: E2 plasma concentration of E-treated males **57**
Table 2: ANOVA: *vtga2* expression of E2-treated males **60**
Table 3: ANOVA: *esr1* expression of E2-treated males **63**
Table 4: Estradiol-induced liver gene expression literature review .. **67**

Chapter 4
Table 1: Sizes and normalization factors of libraries **93**
Table 2: Differentially expressed genes: DE_pop contrast **94**
Table 3: Differentially expressed genes: DE_SDtreat contrast **102**
Table 4: Differentially expressed genes: DE_XEtreat contrast **103**
Table 5: Differentially expressed genes: DE_treat contrast **104**
Table 6: Functional annotation clustering of DE_pop genes **105**

Acknowledgements

To my mentor: Tyrone. “Your life is your story... live it well... tell it well." These words have changed my life. With simply your passion for frogs, you have instilled curiosity and confidence in your students and inspired those who know your story. Thank you for giving me the opportunity to study “life” and for dedicating your life to educating and mentoring us “misfit toys.” I am forever grateful for having you be a part of my story.

To my dissertation committee: Dr. George Bentley and Dr. Lance Kriegsfeld. Thank you for your words of wisdom, critique, and encouragement. You have paved a very impressive road for endocrinologists to come. It is a privilege to know your work and to know you. George makes bomb barbecue ribs and Lance does an excellent rendition of “Sweet Caroline.”

To Hayes Nation: Dr. Sherrie Gallipeau, Dr. Paul Falso, Dr. Theresa Stueve, Lillian Adame, Roya Vahdatinia, Suzuki Rodriguez, Emily Marquez, Stanley Williams III, Jesus Diaz, Mariam Nazir, Mai Nguyen, Win Mai, Dr. Martin Hansen, Rikke Poulsen, Alejandra Cabrera, Flor Gowans, Jae Ha, Christopher Noble, Diana Salazar, Michelle Vy, Brian Tian, Cara He, Faye Pon, Karen Zhang, Maggie Tsang, Nisha Balabhadra, Julia Chow, Maxwell Nguyen, D’andre Buckley, Stephanie Ma, and all other Hayes lab members from past, present, and future. You are the backbone of this dissertation. In addition to the countless hours of tank scrubbing, pipetting, ethanolizing, and frog catching you have contributed to the lab, your personal struggles and successes have inspired and motivated me. I am proud and humbled to have worked hard and played harder alongside the best and brightest. Keep it universal.

To my fellow scientists: Dr. Darcy Ernst, Dr. Nicole Perfito, Daisy Guardado, Dr. Sheng Zhao, Dr. Michihiko Ito, Dr. Shuuji Mawaribuchi, Mikako Wada, Kazuko Fujitani, Dr. Jeremy Crawford, and Dr. Christopher Sifuentes. You all have shown me that knowledge can traverse buildings, campuses, fields of study, borders, and even oceans. Your time, effort, and wisdom are

invaluable, so thank you for contributing to our broader knowledge. I am grateful to be part of a true science community.

To my co-captain: John Wright. Your love, encouragement, and, most importantly, coding skills have helped me complete five of the hardest chapters of my life. I am excited to continue writing many more chapters with you, filled with stories of the sea and tales of the mountains.

To my parents: Anna Luong and Bich Do. Con cám ơn mẹ và bố đã yêu thương và nuôi dưỡng con cho 28 năm nay. Con đi đến bước này tai vì bố mẹ đã hy sinh tất cả mọi thứ cho gia đình. Con thương bố mẹ nhất trên đời và sẽ làm tất cả mọi thứ để làm bố mẹ hãnh diện.

Chapter One

The Importance of Estrogen in Amphibians

PURPOSE and AIMS

This dissertation characterizes differences in response to estrogen exposure between populations of African clawed frogs (*Xenopus laevis*) and aims to elucidate the mechanisms responsible for the observed variation in estrogen susceptibility. Experiments using tadpoles and adult animals were conducted to gain a deeper understanding of the effects of estrogen on distinct developmental stages and tissue types. Chapter One reviews the role of estrogen in amphibian reproduction, the impacts of endocrine disruptors on animal health, and the significance of acknowledging and characterizing response variation in animal models used in scientific studies.

ESTROGEN and AMPHIBIAN REPRODUCTION

Estrogens are a class of steroid hormones ubiquitous and crucial to organismal reproductive success throughout the vertebrate phylum (Lange, Hartel, & Meyer, 2002). They are a product of androgen conversion via the enzyme aromatase (Simpson et al., 1994) and signal physiological changes primarily through intracellular receptors (ERα and ERβ), though estrogen-activated membrane receptors have also been found (Revankar, Cimino, Sklar, Arterburn, & Prossnitz, 2005). There are numerous types of estrogens and estrogen metabolites; estradiol (E2), estrone (E1), and estriol (E3) are the three major endogenous forms in humans (listed in decreasing concentrations and potency). Although it is traditionally considered the "female hormone," E2 is physiologically significant to both females and males and is imperative in gonad development (Guiguen, Fostier, Piferrer, & Chang, 2010; Nakabayashi, Kikuchi, Kikuchi, & Mizuno, 1998), gametogenesis (Eddy et al., 1996; O'Donnell, Robertson, Jones, & Simpson, 2001; Wu, Wang, & Wan, 1993), bone maintenance (Bilezikian, Morishima, Bell, & Grumbach, 1998; Riggs, Khosla, & Melton, 1998), and sex behavior (Davidson, 1969; Ogawa, Lubahn, Korach, & Pfaff, 1997; Simpson & Davis, 2000; Wersinger et al., 1997; Wilczynski & Lynch, 2011). Because of its critical role in estrogen production, aromatase is also implicated in the development of estrogen-dependent cancers (e.g. cancers of the breasts,

endometrium, etc.) (Bergman et al., 2000; Bulun et al., 2007; Lippman, Bolan, & Huff, 1976; Miller, Hawkins, & Forrest, 1982; Yager & Liehr, 1996). Many chemicals that upregulate aromatase, mimic estrogen, or induce stress have contributed to the global decline of aquatic life due to commensurate, deleterious effects on reproductive, developmental, and immune health (Andersen, Vinggaard, Rasmussen, Gjermandsen, & Bonefeld-Jorgensen, 2002; Cevasco et al., 2008; Hayes et al., 2006; Jobling, Nolan, Tyler, Brighty, & Sumpter, 1998; Kazeto, Place, & Trant, 2004; Lutz & Kloas, 1999; Segner et al., 2003). Comparable responses to environmental contaminants have been observed in humans (Cheek, Kow, Chen, & McLachlan, 1999; Colborn, Saal, & Soto, 1993; Ikezuki, Tsutsumi, Takai, Kamei, & Taketani, 2002; Swan, 2006).

Although most amphibians lack discernable sex chromosomes, all amphibians display genetic sex determination (Eggert, 2004). There are few studies that suggest temperature can affect the sex ratios of several species. However, these experimental temperatures were well outside the range of what study animals would experience in the natural environment (Hayes, 1998). Sex chromosomes have been identified in *Xenopus laevis*, where females are heterogametic (ZW) and males are homogametic (ZZ) (Yoshimoto & Ito, 2011). *DM-W*, a W-linked gene and paralog of *DMRT1*, is the female sex determining gene in this species. *DM-W* is transiently and specifically upregulated in the ZW gonad during sex determination at NF stage 50. Expression of *DM-W* results in competitive, antagonistic binding to *DMRT1*-activated, cis-regulatory regions of male-specific genes, leading to suppression of testicular differentiation (Yoshimoto et al., 2010). Simultaneously, *DM-W* increases the expression of *cyp19* (Okada et al., 2009), which codes for P450 aromatase. Ultimately, the increase in estrogen production during sex differentiation leads to ovarian development (Mawaribuchi et al., 2014; Miyashita, Shimizu, Osanai, & Miyata, 2000).

Environment factors, however, may alter sex differentiation in amphibians. Changes in temperature, physical manipulations, and exposure to endocrine-disrupting chemicals can all skew sex ratios in amphibian populations. Researchers, therefore, can readily study the role

of specific environmental factors on development by manipulating rearing conditions of tadpoles. In particular, *X. laevis*, ZZ-tadpoles exposed to estrogen during a critical window during gonad differentiation may become sex-reversed and mature into sexually-functioning ZZ-females (Villalpando & Merchant-Larios, 1990), while exposure to an aromatase or estradiol inhibitor will lead to male phenotype (Miyata & Kubo, 2000). The ability to breed ZZ-females with wild type ZZ-males and obtain all-ZZ-male tadpoles allows for researchers to perform important sex development experiments (Oka et al., 2006).

ENDOCRINE DISRUPTION

Many scientists assert that the world is experiencing a sixth mass extinction and amphibians are bearing the brunt of the weight (Blaustein, Wake, & Sousa, 1994; Wake & Vredenburg, 2008). Amphibians spend a significant proportion of their lives in an aqueous environment; this leaves them more vulnerable to the effects of the environment, including changes to temperature, pH, and exposure to exogenous chemicals. Environmental contaminants are contributing to amphibian decline by disrupting endocrine function; these chemicals are often referred to as endocrine disruptors (EDCs) (Crisp et al., 1998; Diamanti-Kandarakis et al., 2009; Hayes et al., 2006; Tyler, Jobling, & Sumpter, 1998). Estrogenic EDCs may partially feminize males or completely sex-reverse them, leading to a female-dominant sex ratio in the population and detrimental effects for reproduction (Bhandari et al., 2015; Bogi et al., 2003; Hogan, Duarte, Wade, Lean, & Trudeau, 2008; Lambert, Giller, Barber, Fitzgerald, & Skelly, 2015). 4-nonyl-phenol (NP), a compound used in manufacturing household detergents, emulsifiers, and solubilizers, increased vitellogenin (*vtga2*) production, a major estrogen biomarker, in a dose-dependent manner in both male European frogs (*Rana esculenta*) and Italian-crested newts (*Triturus carnifex*) by binding to and activating the estrogen receptor (ER) (Mosconi et al., 2002). Bisphenol A (BPA), a chemical widely used in plastic and epoxy production that can leech into surface and non-surface water, increased the expression of ER, another known estrogen biomarker, feminized the gonads of exposed *X. laevis* tadpoles, and increased the

female-to-male sex ratio of offspring—effects comparable to E2 exposure (Levy, Lutz, Kruger, & Kloas, 2004; Wetherill et al., 2007). Atrazine is a commonly used herbicide that disrupts endocrine function and has feminizing effects across vertebrate species, including fish, amphibians, reptiles, birds, and mammals (including human cells and associated epidemiological effects in humans) (Hayes et al., 2011). Atrazine inhibits phosphodiesterase, resulting in increased cAMP (Roberge, Hakk, & Larsen, 2004). Increased cAMP increases transcription of *cyp19* (Sanderson, Letcher, Heneweer, Giesy, & van den Berg, 2001) and consequently, estrogen production. Furthermore, atrazine exposure can result in complete sex-reversal of genetic male *X. laevis*, resulting in morphological females that lay viable eggs (Hayes et al., 2010).

DIFFERENTIAL RESPONSE TO ESTROGEN and ESTROGENIC EDCS

Few studies have examined variation in susceptibility to xenoestrogens among different rodent strains and even fewer studies have addressed the different vulnerabilities to estrogen or estrogenic compounds among amphibians. Fischer 344 (F344) rats are highly susceptible to diethylstilbestrol- (DES) induced pituitary tumors as compared to Holtzman rats, whose pituitaries did not show significant growth with long term exposure (J. Wiklund, Rutledge, & Gorski, 1981). Holtzman rats have normal alleles at a small number of loci involved in preventing uncontrolled proliferation. The F344 strain is homozygous mutant at these loci and is unable to control DES-induced proliferation. Notably, pituitaries from both rat strains had elevated DNA synthesis with short term E2 treatment. However, this increase persisted in F344 rats, but declined in Holtzman rats with long term treatment, suggesting that F344 rats may be less capable of shutting off E2-stimulated cell proliferation (J. A. Wiklund & Gorski, 1982). BPA stimulated prolactin secretion and DNA synthesis in vaginal epithelium in F344 rats but not in Sprague-Dawley (S-D) rats (Long et al., 2000). There was no difference in BPA metabolism or in binding affinity of estrogen receptor between the two strains. Additionally, low doses of E2 greatly eliminated spermatid maturation, decreased testis weight, and suppressed testis development in male pups from several

strains (C57BL/6J, C17/Jls, S1Jls, E/Jls, and CN-/Jls) as compared to mice of the CD-1 line, which exhibited little or no response to E2 concentrations as much as 16 times higher than the former groups (Spearow et al., 2001). CD-1 individuals are widely used in toxicology and pharmacology research because of their propensity to produce large litter size and vigor. However, selecting for prolificacy is associated with decreased susceptibility to E2. Therefore, researchers are potentially using study animals of a highly resistant phenotype to assess the effects of estrogenic contaminants on wildlife and human health, which may lead to misleading or masked conclusions.

As stated previously, amphibians are facing ongoing global decline and are particularly sensitive to EDCs due to their semi-aquatic life cycle, often aquatic reproduction, and highly permeable skin. *X. laevis* has been selected as the model amphibian because of its prolificacy and sex-specific markers, allowing for molecular sexing. Recently, sex markers have become available for other anuran species, European tree frogs (*Hyla arborea*) and green toads (*Bufo viridis*), and their use in EDC experiments is critical to understanding species-specific differences in EDC vulnerability. Increasing treatment concentrations of 17α-ethinylestradiol (EE2), a potent, synthetic estrogen, increased sex reversal rates in three species. However, *X. laevis* showed higher susceptibility to EE2's effects than both *H. aroborea* and *B. viridis*. For example, exposure to 50 ng/L EE2 resulted in no male-to-female sex reversal in *H. arborea* and *B. viridis*, while 31.3% sex reversal was observed in *X. laevis* (Tamschick, Rozenblut-Koscisty, Ogielska, Lehmann, et al., 2016). Differences in gonad development, snout-to-vent length, weight, and skin coloration in response to the plasticizer BPA have also been studied in these three taxa (Tamschick, Rozenblut-Koscisty, Ogielska, Kekenj, et al., 2016). Thus, the use of one model organism in toxicology experiments should be done with caution and results from such studies may not accurately represent outcomes in other anuran taxa.

CONCLUSIONS

Estrogen is critical to the reproductive success of all vertebrates, playing a role in early development and having persistent effects on adult reproductive behavior and function. Estrogenic EDCs can cause changes to gene expression, morphology, and physiology of exposed wildlife as well as of humans. However, there is a marked lack of studies that address variation in susceptibility to estrogen in animal models used in EDC experiments. This dissertation characterizes the differences in response to estrogen exposure in the African clawed frog (*Xenopus laevis*) and aims to elucidate the mechanisms responsible for the observed variation in estrogen susceptibility.

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Chapter Two

Characterization of Differential Estrogen Susceptibility in Populations of African Clawed Frogs

ABSTRACT

We examined how two source populations of *Xenopus laevis* display different susceptibilities to the feminizing effects of 17-β estradiol (E2). All-male, ZZ-tadpoles from a commercial source, Xenopus Express (XE), and from a feral population, San Diego (SD), were treated with 3.00 ng/ml E2 from 10 days post-hatch until metamorphosis. Animals were then sacrificed, gonadal sex was determined via dissection, and sex ratios were calculated. Experiments were conducted with multiple parents within each population as well replicated breeds from previously used parents (familial replicates) in order to verify sex reversal trends. There were no significant differences in sex ratios among breeds within a population. However, with E2 treatment, SD breeds yielded greater numbers of ZZ-females than XE breeds. Additionally, familial replicates from each population, XE4 and SD4, did not yield significantly different sex ratios within the populations, but SD4 breeds had significantly higher ZZ-female percentages than XE4. These data indicate that there are inherent differences in gonad susceptibility to the feminizing effects of E2 within a species and implied varying population susceptibility to the feminizing effects of endocrine disruptors.

INTRODUCTION

Sex is determined genetically in *Xenopus laevis*; females are heterogametic (ZW), while males are homogametic (ZZ) (Yoshimoto & Ito, 2011). However, environment factors can alter genetic sex differentiation in this species. Variations in temperature, physical manipulations, and exposure to endocrine disrupting chemicals (EDCs) can all skew sex ratios in amphibians (Hayes, 1998; Hogan, Duarte, Wade, Lean, & Trudeau, 2008; Nakamura, 2013). Thus, researchers can readily study the effects of environmental factors on development by manipulating the rearing conditions of tadpoles. In particular, male tadpoles raised in the presence of E2 may be sex-reversed and differentiate into sexually-functional, ZZ-females (Miyata, Koike, & Kubo, 1999). The ability to generate all-male offspring by breeding ZZ-females with wild type males, thus eliminating

the W chromosome, serves as an invaluable tool to study the feminizing effects of estrogenic EDCs (Oka et al., 2006).

Notably, there are many known environmental contaminants that are either estrogenic themselves or induce aromatase, the enzyme that converts androgens to estrogens (Fan et al., 2007; Kazeto, Place, & Trant, 2004; Kloas, Lutz, & Einspanier, 1999; Nimrod & Benson, 1996; Tompsett et al., 2015). For example, ZZ-male, *X. laevis* tadpoles have been sex-reversed into functional, morphological females by treatment with atrazine, a commonly used herbicide that upregulates aromatase (Hayes et al., 2010). Additionally, treatment with 17α-ethinylestradiol (EE2) increases mixed sex and abnormal gonad phenotypes in male tadpoles (Tompsett et al., 2012), while bisphenol A also has the ability to alter amphibian sex ratios towards female and increases estrogen receptor transcription (Levy, Lutz, Kruger, & Kloas, 2004).

To better understand these extreme outcomes, it is important to study the role of E2 during sex differentiation. We characterized differing susceptibilities to the sex reversing effects of E2 of two different source populations of *X. laevis*. Experiments were conducted with multiple parents within each population as well replicated breeds from previously used parents in order to verify sex reversal trends.

MATERIALS and METHODS

Animal breeding

Adults animals were from either a feral colony originating from San Diego, California (SD) or from a commercial colony, purchased from Xenopus Express (XE) (Brooksville, FL, USA). Both colonies were maintained at the University of California, Berkeley. Several parents were bred from each population and multiple breeds from a single pair were also performed (familial replicates). Four XE wild type males (XEZZM: 36, 1110, 1616, 2356), three XE ZZ-females (XEZZF: 594, 793, 2711), four SD wild type males (SDZZM: 18, 134, 140, 699), and four SD ZZ-females (SDZZF: 167, 384, 385, 1615) were used for breeding (Table 1). For all

experiments, females and males were injected with 800 IU and 400 IU human choriogonadotropin (Sigma-Aldrich, St. Louis, MO, USA), respectively, at 16:00 and allowed to breed overnight.

Larval care

Embryos were separated from the parents at 9:00 the following day (day 1) and aerated. Tadpoles were fed a solution of ground rabbit chow (Purina, Minneapolis, MN, USA) daily starting on day 4. At day 7, the larvae were netted into tanks, five animals at a time repeatedly, until all tanks contained 30 larvae. Larvae were raised in four liters of aerated 10% Holtfreter's solution. Food levels were adjusted as the animals grew to maximize growth.

Dosing

Treatment was added to the tanks starting on day 10. Experiments used 3.00 ng/ml E2, a concentration shown to effectively sex-reverse animals (Oka et al., 2006). All stock solutions were made in 10 ml ethanol, mixed in 5-gallon containers, and dispensed into treatment tanks; all tanks contained 0.003% ethanol. Final E2 concentrations were confirmed by radioimmunoassay (RIA). Water and treatments were renewed once every 72 hours. For all experiments, each treatment group was replicated three times with 30 animals per replicate (total of 90 animals per treatment). Tanks were systematically rotated around the shelf every three days to ensure that no one treatment nor one tank experienced positional effects. Experiments were carried out at 22° C and under a 12-h/12-h light/dark cycle. Animals were exposed throughout the entire larval period, from 10 days post-hatch (dph) until complete tail reabsorption (NF stage 66). All treatment exposure was conducted blindly with color-coded treatment containers and tanks.

Sex genotyping

To verify genetic sex of the parents and a sample of the offspring, genomic DNA was isolated from toe tips of animals and prepared by tissue lysis with proteinase K (Sigma-Aldrich, St. Louis, MO, USA) digestion. The

ZW genotype was determined by multiplex PCR amplification (37 cycles) of *DM-W* (W-specific), and *DMRT-1* (Z-specific) was also amplified as a control. The primers used were (Yoshimoto et al., 2008):

DM-W-forward 5′-CCACACCCAGCTCATGTAAAG-3′
DM-W-reverse 5′-GGGCAGAGTCACATATACTG-3′
DMRT1-forward 5′-AACAGGAGCCCAATTCTGAG-3′
DMRT1-reverse 5′-AACTGCTTGACCTCTAATGC-3′

All primers were ordered from Integrated DNA Technologies (Coralville, IA, USA). The PCR products were 260 basepairs (*DM-W*) and 206 basepairs (*DMRT-1*). Ethidium bromide–labeled PCR products were visualized under UV light after electrophoresis on a 1.2% agarose gel.

Morphological sexing

Phenotypic sex of all individuals was determined based on gross gonadal morphology using a Nikon SMZ 10A dissecting scope (Technical Instruments, Burlingame, CA, USA). Histological analysis was conducted on any animals for which the sex was ambiguous when determined by dissection. For this, the gonad-kidney complex was dissected out and dehydrated in graded alcohols, followed by infiltration with histoclear (National Diagnostics, Atlanta, GA, USA) and paraffin (Fisher Scientific, Hampton, NH, USA). Sections were cut at 8 μm and stained in hematoxylin and eosin (Fisher Scientific, Hampton, NH, USA). Sections were then examined under a microscope and sex was determined based on histological morphology. All analyses were conducted blindly.

Statistics

Statistical analysis was performed in Minitab Express 1.5.0 (Minitab Inc., State College, PA, USA) using the Kruskal-Wallis test. Values of 0 were expressed as 0.0001 for analysis purposes.

RESULTS

Sex ratios

No females were found in control groups of either population. In response to E2 treatment, the XE1 breed yielded 0 ZZ-females out of 71 animals (0.0% females), the XE2 breed yielded 5 ZZ-females out of 55 animals (9.1% females), and the XE3 breed yielded 21 ZZ-females out of 67 animals (31.3% females) (Figure 1A). There were no significant differences in sex ratios among breeds XE1-XE3 (Kruskal-Wallis, *p*-value = 0.3679) and when combined (XE), no significant difference was observed between XE-CON and XE-E2 (Kruskal-Wallis, *p*-value = 0.5066) (Figure 1B). In response to E2 treatment, the SD1 breed yielded 26 ZZ-females out of 59 animals (44.1% females), the SD2 breed yielded 76 out of 79 animals (96.2% females), and the SD3 breed yielded 48 out of 57 animals (84.2% females) (Figure 1A). There were no significant differences in sex ratios among breeds SD1-SD3 (Kruskal-Wallis, *p*-value = 0.3679) and when combined (SD), there was a significant difference between SD-CON and SD-E2 (Kruskal-Wallis, *p*-value = 0.0463) (Figure 1B). Notably, there was a significant difference between the average XE-E2 sex ratio and SD-E2 sex ratio (Kruskal-Wallis, *p*-value = 0.0495).

With E2 treatment, the XE familial replicates, XE4a, XE4b, and XE4c, yielded 8 females out of 62 animals (12.9% females), 14 females out of 56 animals (25.0% females), and 5 females out of 73 animals (6.8% females), respectively (Figure 2A). The sex ratios among these breeds were not significantly different (Kruskal-Wallis, *p*-value = 0.3679), and when combined (XE4), there was a significant difference between XE4-CON and XE4-E2 (Kruskal-Wallis, *p*-value = 0.0463) (Figure 2B). With E2 treatment, the familial replicates, SD4a, SD4b, and SD4c, yielded 51 females out of 58 animals (87.9% females), 32 females out of 49 animals (65.3% females), and 32 females out of 46 animals (69.6% females), respectively (Figure 2A). The sex ratios among these breeds were not significantly different (Kruskal-Wallis, *p*-value = 0.3679), and when combined (SD4), there was a significant difference between SD4-CON and SD4-E2 (Kruskal-Wallis, *p*-value = 0.0463) (Figure 2B). Notably, there was a significant difference between the average XE4-E2 sex ratio and the average SD4-E2 sex ratio (Kruskal-Wallis, *p*-value = 0.0495) (Figure 2B).

DISCUSSION

Due to their ease of maintenance in the laboratory, year-round production of numerous, large, robust eggs, long life spans, and genetic similarity to humans, *Xenopus* has been established as the amphibian model organism (Cannatella & Desa, 1993). Early experiments achieved sex-reversal in *X. laevis* (Chang & Witschi, 1955) and proved this species to be a valuable tool in studying estrogenic EDCs (Bogi et al., 2003; Huang, Matthews, Fertuck, & Zacharewski, 2005; Lutz & Kloas, 1999; Oka et al., 2006). Current literature addresses differences in susceptibility to E2 among anuran taxa. *Bombina bombina* and *Bombina variegata*, two closely-related species of fire-bellied toad, and *X. laevis* tadpoles show higher susceptibility to E2 compared to tadpoles of *Hyla arborea*, the European tree frog, and *Bufo viridis*, the European green toad (Piprek, Pecio, Kubiak, & Szymura, 2012). Studies examining EE2 confirmed higher susceptibility in *X. laevis* than in *H. arborea* and *B. viridis* (Tamschick et al., 2016). The difference in E2 or estrogenic EDC susceptibility has been observed in other organisms such as mouse, rat, flounder, chicken, and breast cancer cell lines (Astiningsih & Rogers, 1996; Bailey & Nephew, 2002; Kirby et al., 2006; Limer, Parkes, & Speirs, 2006; Long et al., 2000; Spearow et al., 2001; Yamasaki, Sawaki, & Takatsuki, 2001).

This current experiment is one of two available studies addressing differences in estrogen susceptibility found within an amphibian species. The other is a recent publication, which examined *Glandirana rugosa*, the Japanese wrinkled frog, a geographically diversified species that shows regional differences in sex determination and sex chromosome morphology. E2 treatment resulted in ZW populations with sex ratios that significantly deviated from 1:1, while sex ratios remained unchanged in XY populations. Among the ZW populations, the Kanazawa and Niigata populations showed different critical periods for E2-induced sex reversal (Miura, Ohtani, Ogata, & Ezaz, 2016). Our results indicate inherent differences in gonad susceptibility to the feminizing effects of E2 between two source populations of *X. laevis*, XE and SD, and imply varying susceptibility to the feminizing effects of endocrine disruptors. At a given

E2 concentration, SD populations yielded greater numbers of morphological females than XE populations, and was thus defined as an E2-susceptible population and XE, an E2-resistant population. These findings were consistent for experiments using tadpoles from different parent pairs from each population and experiments testing familial replicates.

The characterization of these populations is valuable for researchers who may unwittingly be using *X. laevis* with extreme susceptibilities to test the effects of E2 or other forms of estrogen. A review of the literature (Table 2) showed that past studies on the effects of E2 on sex reversal published drastically disparate results with similar ranges of treatment (Kloas et al., 2009; Miyata & Kubo, 2000; Pickford, Hetheridge, Caunter, Hall, & Hutchinson, 2003; Sharma & Patino, 2010; Wolf et al., 2010). Notably, with the exception of two other studies, this current experiment was the only one to use all-ZZ males to examine sex reversal. The use of mixed ZZ and ZW groups for such studies resulted in ambiguous findings as it is not possible to distinguish ZZ-females resulting from E2 treatment from wild type ZW-females without extensive genotyping. We used the following equation to approximate the number of males sex-reversed by E2 in studies with mixed ZZ and ZW animals:

$$\frac{\%\ females\ in\ treatment\ -\ \%\ females\ in\ control}{\%\ males\ in\ control}$$

When researchers did report the source of their study animals, several groups used frogs purchased from Xenopus Express (Homosassa, FL, USA), the population we have characterized as E2-resistant. For example, at 10^{-8} M E2 resulted in ~55% (Kloas et al., 1999), ~68% (Bogi, Levy, Lutz, & Kloas, 2002), ~71%(Levy et al., 2004), 100% (Oka et al., 2008), and from this current study, 13.5% ZZ-females. Higher treatment at 10^{-4} M E2 resulted in 100% (Hayes et al., 2006; Hu, Smith, & Carr, 2008; Miyata et al., 1999; Qin, Zhou, Chu, & Xu, 2003), 36% (Carr et al., 2003), and 0% ZZ-females (Coady et al., 2005). Our results offer an explanation to the major inconsistencies in reported effects of estrogenic EDCs, such as atrazine, on amphibian life

(Hayes et al., 2011; Solomon et al., 2008). Notably, it would behoove scientists to study EDCs using the most vulnerable populations as they serve as sensitive bioindicators while resistant populations may yield outcomes that are not representative of prevalent physiological effects.

Further investigation to confirm this difference in population E2-susceptibility is needed. Because all sex differentiation is achieved through changes in gene regulation, it is evident that the next steps are to explore gene expression differences between developing gonads of E2-treated XE and SD tadpoles using molecular techniques such as quantitative reverse transcription polymerase chain reaction (qRT-PCR) and RNA sequencing (RNA-Seq). Additionally, a reliable indicator of E2 exposure is the upregulation of vitellogenin and estrogen receptor genes in adult *X. laevis* liver (Lutz, Blodt, & Kloas, 2005; Selcer, Williams, & Skoloda, 2003; Sumpter & Jobling, 1995). Testing differences in gene response between XE and SD adults would address whether or not disparities in E2-susceptibility span tissue types and developmental stages and if the basis of susceptibility is transcription-based. Other avenues to explore are differences in population E2 metabolism and *in vitro* studies of tissue-specific E2 response.

It is crucial that researchers fully characterize and understand their animal model of choice. Using a model species, albeit valuable in many cases, may result in misleading results that do not represent the taxa. Additionally, further consideration must be taken when choosing populations or strains of a species as we have demonstrated vast disparities in their morphological and response to E2.

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Population	Male IDs	*n*	Female IDs	*n*
Xenopus Express	XEZZM: 36, 1110, 1616, 2356	4	XEZZF: 594, 793, 2711	3
San Diego	SDZZM: 18, 134, 140, 699	4	SDZZF: 167, 384, 385, 1615	4

Breed name	Breed parents	Control # M, # ZZ-F	3.0 ng/ml E2 # M, # ZZ-F
XE-1	XEZZM1110 x XEZZF793	74 M, 0 F	71 M, 0 F
XE-2	XEZZM36 x XEZZF2711	63 M, 0 F	50 M, 5 F
XE-3	XEZZM2356 x XEZZF2711	80 M, 0 F	46 M, 21 F
XE-4a	XEMZZ1616 x XEZZF594	78 M, 0 F	54 M, 8 F
XE-4b	"	75 M, 0 F	42 M, 14 F
XE-4c	"	83 M, 0 F	68 M, 5 F
SD-1	SDZZM699 x SDZZF384	79 M, 0 F	33 M, 26 F
SD-2	SDZZM134 x SDZZF1615	73 M, 0 F	3 M, 76 F
SD-3	SDZZM18 x SDZZF385	58 M, 0 F	9 M, 48 F
SD-4a	SDZZM140 x SDZZF167	54 M, 0 F	7 M, 51 F
SD-4b	"	72 M, 0 F	17 M, 32 F
SD-4c	"	56 M, 0F	14 M, 32 F

Table 1 Breed information. Shown are every breed and the parents involved. Also listed are the resulting number of males and sex-reversed males, ZZ-females, for both control and E2-treated groups.

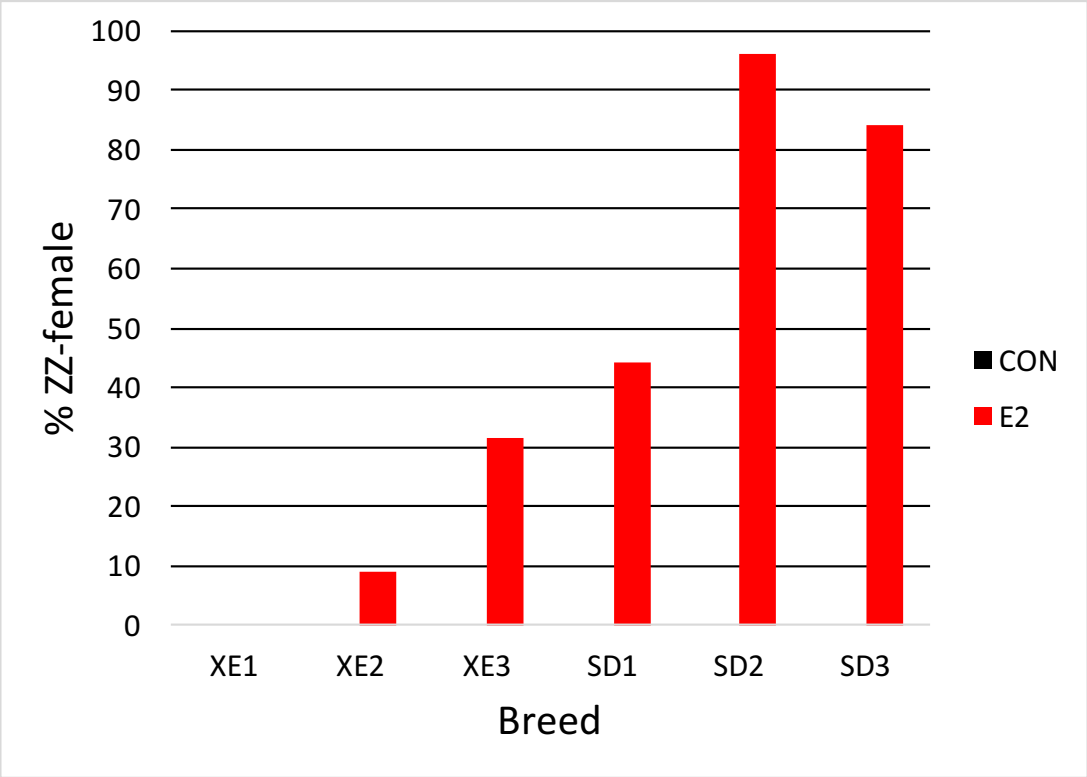

Figure 1A Breed-wise ZZ-female percentages. Shown are the percentages of ZZ-females resulting from treatment of tadpoles with 3.00 ng/ml E2. For both populations, control breeds yielded no females. In response to E2 treatment, the XE1 breed yielded 0 ZZ-females out of 71 animals (0.0% females), the XE2 breed yielded 5 ZZ-females out of 55 animals (9.1% females), and the XE3 breed yielded 21 ZZ-females out of 67 animals (31.3% females). There were no significant differences in sex ratios among breeds XE1-XE3 (Kruskal-Wallis, *p*-value = 0.3679). In response to E2 treatment, the SD1 breed yielded 26 ZZ-females out of 59 animals (44.1% females), the SD2 breed yielded 76 out of 79 animals (96.2% females), and the SD3 breed yielded 48 out of 57 animals (84.2% females). There were no significant differences in sex ratios among breeds SD1-SD3 (Kruskal-Wallis, *p*-value = 0.3679).

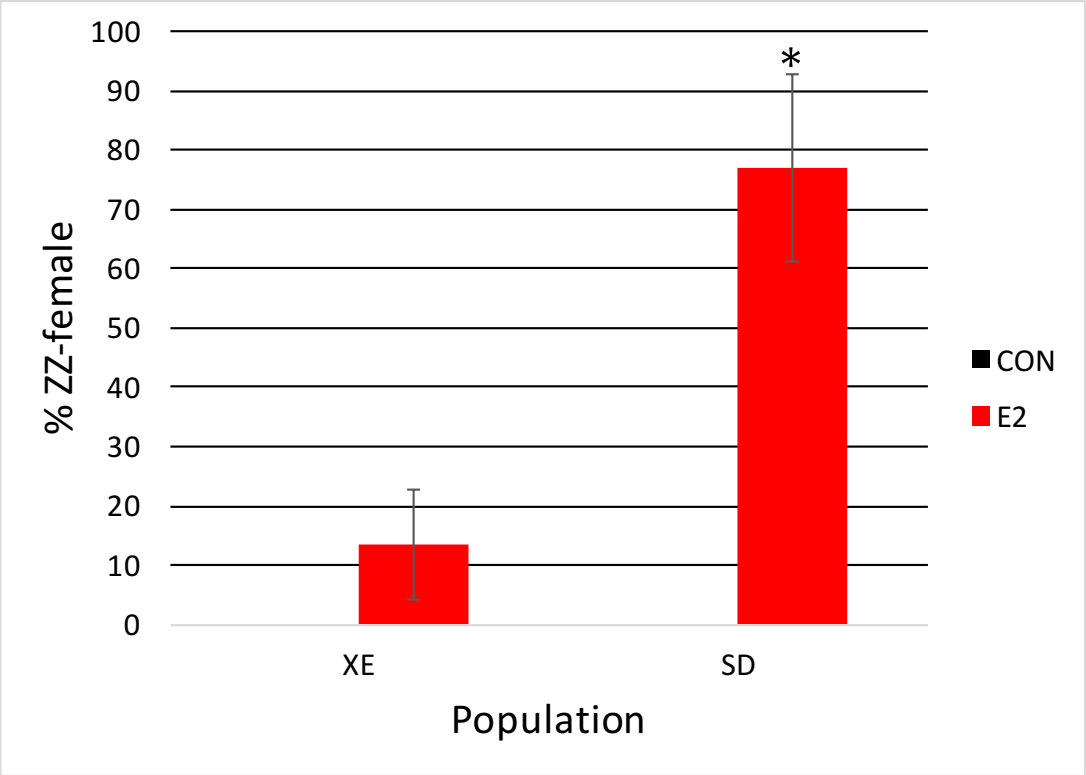

Figure 1B Average ZZ-female percentages. Shown are the average percentages of ZZ-females resulting from treatment of tadpoles with 3.00 ng/ml E2. XE breeds (XE1-XE3, n = 3) were consolidated and SD breeds (SD1-SD3, n = 3) were consolidated to obtain average ZZ-female percentages. No significant difference was observed between control groups and XE-E2 (Kruskal-Wallis, p-value = 0.5066). Notably, there were significant differences between control groups and SD-E2 (Kruskal-Wallis, p-value = 0.0463) and between the average XE-E2 sex ratio and the average SD-E2 sex ratio (Kruskal-Wallis, p-value = 0.0495). Asterisks (*) indicate significance.

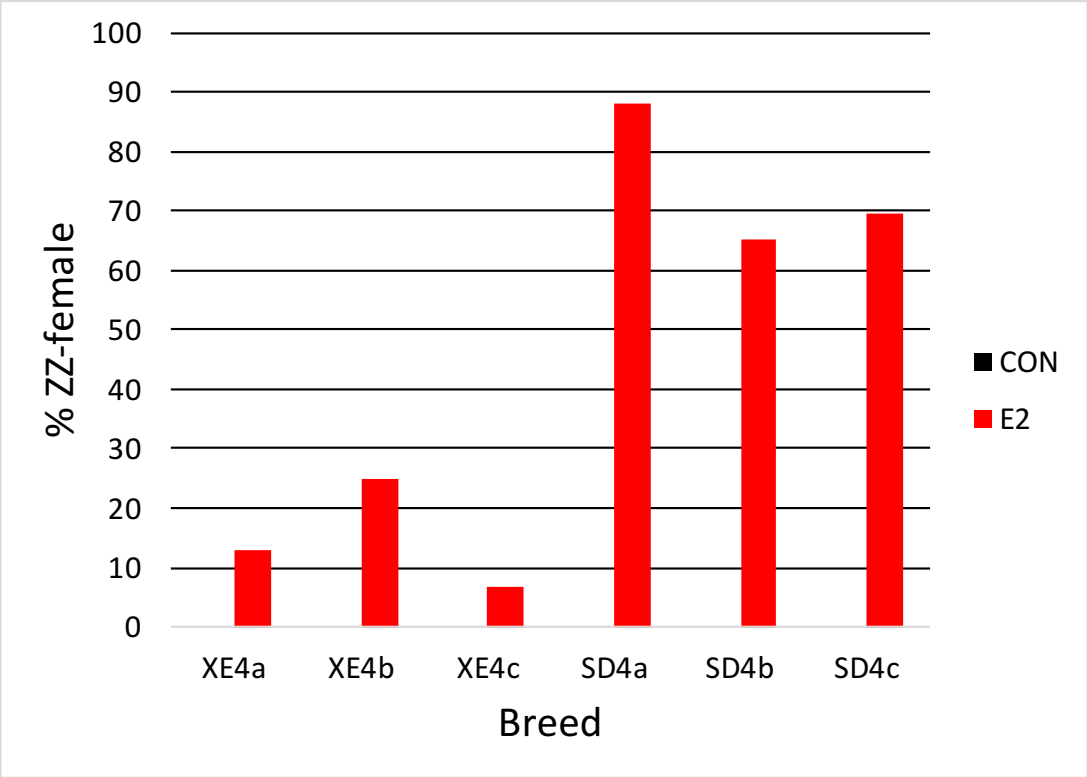

Figure 2A Familial replicates: breed-wise ZZ-female percentages. Shown are the percentages of ZZ-females resulting from treatment of tadpoles with 3.00 ng/ml E2. For both XE and SD, control breeds yielded no females. With E2-treatment, XE familial replicates, XE4a, XE4b, and XE4c, yielded 8 females out of 62 animals (12.9% females), 14 females out of 56 animals (25.0% females), and 5 females out of 73 animals (6.8% females), respectively. With E2-treatment, SD familial replicates, SD4a, SD4b, and SD4c, yielded 51 females out of 58 animals (87.9% females), 32 females out of 49 animals (65.3% females), and 32 females out of 46 animals (69.6% females), respectively. The sex ratios from these replicate breeds were not significantly different within each population (Kruskal-Wallis, p-value = 0.3679).

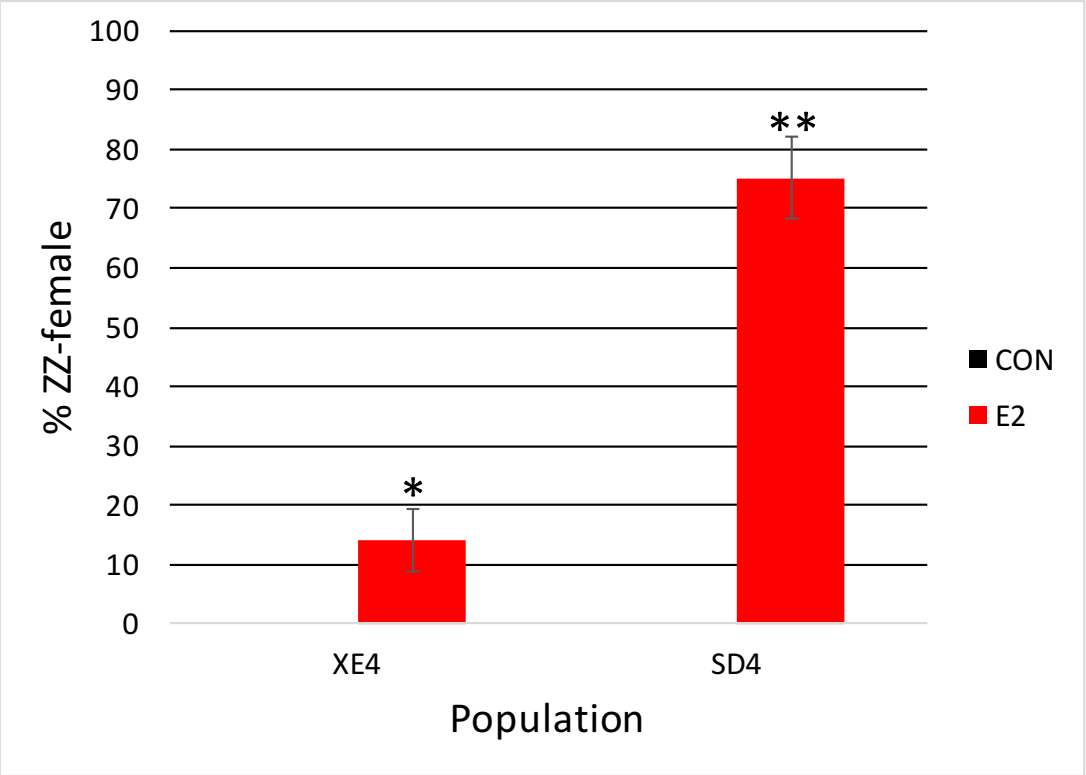

Figure 2B Familial replicates: average familial ZZ-female percentages. Shown are the percentages of ZZ-females resulting from treatment of tadpoles with 3.00 ng/ml E2. Data from XE familial replicate breeds (XE4a-XE4c, n = 3) were consolidated and from SD familial replicate breeds (SD4a-SD4c, n = 3) were consolidated to obtain average ZZ-female percentages. There was a significant difference among control groups, XE4-E2, and SD4-E2 (Kruskal-Wallis, p-value = 0.0463). Notably, there was a significant difference between the average XE4 sex ratio and the average SD4 sex ratio (Kruskal-Wallis, p-value = 0.0495). Asterisks (*) indicate significance.

Year	Author(s)	Treatment(s)	Method	Treatment duration	Species	Population	Results (% ZZ-females of total males)
1990	Villalpando & Merchant-Larios	10^{-4} M estradiol benzoate	*In vivo* – immersion	Stages 44-50 (~4-15 dph) – metamorphosis	*Xenopus laevis* – mixed ZZ and ZW	Not determined	100% ZZ-females
1999	Miyata et al.	10^{-4} M E2	*In vivo* – immersion	Stages 48, 49 or 50 – stages 51 and 61	*Xenopus laevis* – mixed ZZ and ZW	Not determined	100% ZZ-females for all treatment stages except 48-51
1999	Kloas et al.	10^{-8} M and 10^{-7} M E2	*In vivo* – immersion	3 dph - metamorphosis	*Xenopus laevis* – mixed ZZ and ZW	Department of Zoology II, University of Karlsruhe	At 10^{-8} M: ~55% ZZ-females At 10^{-7} M: ~97% ZZ-females
2000	Miyata & Kubo	3.6×10^{-9} – 3.6×10^{-7} M E2	*In vitro* – organ culture	Stage 50 (~15 dph) – 14 days later	*Xenopus laevis* – mixed ZZ and ZW	Not determined	At 3.6×10^{-9} M: ~41% ZZ-ovaries At 3.6×10^{-8} M: ~61% ZZ-ovaries At 3.6×10^{-7} M: ~82% ZZ-ovaries
2002	Bogi et al.	10^{-8} M & 10^{-7} M E2	*In vivo* – immersion	3 dph – metamorphosis	*Xenopus laevis* – mixed ZZ and ZW	Department of Inland Fisheries, Leibniz-Institute of Freshwater Ecology and Inland Fisheries	At 10^{-8} M: ~68% ZZ-females At 10^{-7} M: ~96% ZZ-females
2003	Qin et al.	10^{-4} M E2	*In vivo* – immersion	Stage 46/47 (~4-5 dph) – stage 61/62 (~48-49 dph)	*Xenopus laevis* – mixed ZZ and ZW	Institute of Developmental Biology of the Chinese Academy Sciences	100% ZZ-females
2003	Pickford et al.	2.3×10^{-6} M E2	*In vivo* – immersion	2 dph - metamorphosis	*Xenopus laevis* – mixed ZZ and ZW	Not determined	~37% ZZ-females
2003	Carr et al.	10^{-4} M E2	*In vivo* – immersion	48 hph – 80 dph	*Xenopus laevis* – mixed ZZ and ZW	Xenopus Express (Homosassa, FL, USA)	~36% ZZ-females
2004	Levy et al.	10^{-8} M & 10^{-7} M E2	*In vivo* – immersion	Stage 42/43 (~ 3 dph) – 120 days later	*Xenopus laevis* – mixed ZZ and ZW	Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany	At 10^{-8} M: ~71% ZZ-females At 10^{-7} M: ~66% ZZ-females; ~92% ZZ-females
2005	Coady et al.	10^{-4} M E2	*In vivo* – immersion	72 hph – metamorphosis	*Xenopus laevis* – mixed ZZ and ZW	Xenopus Express (Homosassa, FL, USA)	0% ZZ-females

Year	Author(s)	Treatment(s)	Method	Treatment duration	Species	Population	Results (% ZZ-females of total males)
2006	Hayes et al.	10^{-4} M E2	*In vivo* – immersion	Stage 50 – 1 week later, 2 weeks later, 49 days later	*Xenopus laevis* – mixed ZZ and ZW	Not determined	1 week treatment: ~60% ZZ-females 2 weeks treatment: ~92% ZZ-females 49 days treatment: 100% ZZ-females
2006	Oka et al.	10^{-10} M – 2 x 10^{-8} M E2	*In vivo* – immersion	Stage 49/50 (~12-15 dph) – stage 57 (~47 dph)	*Xenopus laevis* – all ZZ-males	Not determined	At 10^{-10} M: 0% ZZ-females At 10^{-9} M: 96.7% ZZ-females At 10^{-8} M: 100% ZZ-females At 2 x 10^{-8} M: 100% ZZ-females
2008	Hu et al.	10^{-6} M – 10^{-4} M E2	*In vivo* – immersion	24 hpf – metamorphosis	*Xenopus laevis* – mixed ZZ and ZW	Xenopus Express (Homosassa, FL, USA)	At 10^{-6} M: ~39% ZZ-females At 10^{-5} M: ~98% ZZ-females At 10^{-4} M: 100% ZZ-females
2008	Oka et al.	10^{-9} M E2	*In vivo* – immersion	Stage 49 (~12 dph) – metamorphosis	*Xenopus laevis* – all ZZ-males	Yamamura frog store (Hiroshima, Japan)	72.4% ZZ-females
2009	Kloas et al.	2 x 10^{-7} M E2	*In vivo* – immersion	8 dpf – metamorphosis /83 dpf	*Xenopus laevis* – mixed ZZ and ZW	Xenopus I (Dexter, MI, USA)	Experiment 1: ~50% ZZ-females Experiment 2: ~15% ZZ-females
2010	Wolf et al.	0.2 – 6.0 x 10^{-6} M E2	*In vivo* – immersion	8 dpf – metamorphosis	*Xenopus laevis* – mixed ZZ and ZW	Xenopus I (Dexter, MI, USA)	At 0.2 x 10^{-6} M E2: ~65, 50, 14% % ZZ-females At 1.5 x 10^{-6} M E2: ~81% ZZ-females At 6.0 x 10^{-6} M E2: ~95% ZZ-females
2010	Sharma et al.	10^{-6} M E2	*In vivo* – immersion	24 hpf - metamorphosis	*Xenopus laevis* – mixed ZZ and ZW	Xenopus Express (Homosassa, FL, USA)	~30% ZZ-females
2016	Luong et al.	1.1 x 10^{-8} M E2	*In vivo* – immersion	10 dph – metamorphosis	*Xenopus laevis* – all ZZ-males	Xenopus Express (Homosassa, FL, USA) & San Diego (San Diego, CA, USA)	XE: 13.5% ZZ-females SD: 76.9% ZZ-females

Table 2 Estradiol-induced sex reversal literature review. Above are summaries of notable papers that examined estradiol- (E2) induced male-to-female sex reversal in *X. laevis*. All staging was done using the Nieukwoop-Faber method. Terminology: hours post-fertilization (hpf), days post-fertilization (dpf), days post-hatch (dph). If studies were conducted with mixed ZZ and ZW tadpoles, % ZZ-females of total males was approximated by the following equation: $\frac{\%\ females\ in\ treatment - \%\ females\ in\ control}{\%\ males\ in\ control}$

Chapter Three

Differential Estrogen Susceptibility Spans Tissue Types and Developmental Stages

ABSTRACT

Male tadpoles from two different *Xenopus laevis* populations show drastically different sex reversal rates in response to estradiol (E2) treatment. We examined the response of livers to E2 in adult, male frogs in order to investigate possible mechanisms that underlie variation in E2 susceptibility. The liver of adult *X. laevis* contains E2 receptors (*esr1*) and responds to E2 exposure with robust increases in vitellogenin (*vtga2*) and *esr1* production. Because of this direct interaction and the abundance of tissue available, the liver is an effective model to test E2 susceptibility. Males from E2-susceptible (SD) and E2-resistant (XE) populations were treated with 0.00, 0.03, 0.30, 3.00, 30.00, and 300.00 ng/ml E2 for 12 hours, a duration chosen as the result of an initial time course study. Plasma E2 levels and gene expression were measured via radioimmunoassay (RIA) and quantitative reverse transcription polymerase chain reaction (qRT-PCR), respectively. SD males had higher plasma E2 levels than XE males for all E2 treatments and treatment increased *vtga2* expression for both populations. SD animals had greater fold change in *vtga2* than XE at all E2 concentrations. However, the change was not directly proportional to concentration. For both populations, *esr1* increased with increasing E2 treatment except for a drastic decrease at the highest concentration. This indicated a non-monotonic relationship between *vtga2* and E2 concentration. XE males, however, showed greater fold changes in *esr1* expression than SD animals. *vtga2* was correlated to E2 plasma levels in both populations while *esr1* showed strong correlation to plasma E2 levels only when omitting the large decrease in gene expression at 300.00 ng/ml E2. There are inherent differences in the susceptibility to E2, which were observed in sex differentiation of the tadpole gonad and *vtga2* expression in the adult liver. Difference in E2 susceptibility was observed at the transcription level and spans tissue types and developmental stages.

INTRODUCTION

We demonstrated population-dependent susceptibility to the feminizing effects of E2 in tadpole gonads and implied varying

susceptibility to endocrine disruptors. Here, we examined the response of livers to E2 in adults to investigate mechanisms that underlie variation in susceptibility. Cytosolic and nuclear estrogen receptors (*esr1*) are present in the hepatocytes of the adult *X. laevis* liver (Hayward, Brock, & Shapiro, 1982). Female livers will bind endogenous E2 synthesized by the ovaries and, in response, produce vitellogenin (*vtga2*), a precursor to the egg yolk proteins phosvitin and lipovitellin. Vitellogenin is then transported in the blood and absorbed by developing oocytes. Inducibility of *vtga2* first occurs at the end of pro-metamorphosis and increases during the completion of metamorphosis (Knowland, 1978). Although male *X. laevis* do not typically produce high enough E2 levels to induce *vtga2* synthesis, exposure to exogenous E2 will cause adult male livers to synthesize and secrete vitellogenin (May & Knowland, 1981). Moreover, there are no significant differences in ligand affinities to *esr1* observed between the male and female liver. Because of this direct interaction and the abundance of tissue available, the liver is an effective model that can be used when measuring an animal's physiological response to E2 and estrogenic endocrine disruptors (EDCs) (Brock & Shapiro, 1983; Lutz & Kloas, 1999; Mosconi et al., 2002; Palmer, Huth, Pieto, & Selcer, 1998; Sanderson, Letcher, Heneweer, Giesy, & van den Berg, 2001; Selcer, Williams, & Skoloda, 2003; Sumpter & Jobling, 1995; Tompsett et al., 2015) and therefore, testing E2 susceptibility. We defined liver E2 susceptibility by the quantity of *vtga2* and *esr1* transcripts resulting from E2 exposure—the higher the expression, the greater the susceptibility.

METHODS AND MATERIALS

Animals

Adult males characterized as E2-susceptible (SD) were from a captive colony originating from San Diego, California and maintained at the University of California, Berkeley. E2-resistant (XE), adult males were from lab-bred colonies, whose original parents were purchased from Xenopus Express (Brooksville, FL, USA). Animals were weighed and then randomly assigned to each treatment.

Time-course experiment

Frogs were introduced to four liters of 10% Holtfreter's solution with a concentration of 3.00 ng/ml E2 (1.1×10^{-8} M). Animals were exposed for either 0 (baseline), 3, 6, 12, 18, or 24 hours. All stock solutions were made in 10 ml ethanol, mixed in 5-gallon containers, and dispensed into treatment tanks; all tanks contained 0.003% ethanol. Final E2 concentrations were confirmed by radioimmunoassay (RIA). Experiments were carried out at 22° C under a 12-h/12-h light/dark cycle. All exposures were conducted blindly with color-coded treatment containers and tanks. Each treatment was replicated five times in each population.

At termination of each exposure duration, animals were removed and blood was collected via cardiac puncture with heparin-coated (Sigma-Aldrich, St. Louis, MO, USA) 26G ½ needles (Becton Dickinson, Franklin Lakes, NJ, USA) and 1 ml syringes (Fisher Scientific, Hampton, NH, USA). At least 0.5 ml of blood was drawn from each male. Animals were pithed and opened ventrally. A piece of liver about 60 mg was dissected out and flash-frozen in liquid nitrogen. Tissues were stored at -80° C until RNA extraction. Samples of treatment water were taken prior to introduction of animals and after 12 hours of exposure.

Concentration-response experiment

Concentration response to estrogen was examined at 0.03, 0.30, 3.00, 30.00, and 300.00 ng/ml (1.1×10^{-10} M - 1.1×10^{-6} M, respectively) by exposing frogs for 12 hours. A vehicle control of 95% ethanol was included. Treatments were made and verified as described above and rearing conditions were kept constant. Each treatment was replicated five times for each population. At 19:00, animals were placed in four liters of treatment water for 12 hours. At 7:00 the following day, animals were removed from treatment and blood and tissue samples were collected as described above. Water samples were taken prior to introduction of animals and after 12 hours of immersion.

Radioimmunoassay (RIA)

Blood was immediately placed on ice and centrifuged at 1,000 RPM for 5 minutes at 3° C. Plasma was extracted from the samples via glass Pasteur pipettes and stored at -12° C until assayed. Steroid hormones from the aqueous phase were extracted with diethyl either (Sigma-Aldrich, St. Louis, MO, USA). Samples were dried down under nitrogen gas and reconstituted with phosphate buffered saline with gelatin (PBS-G). E2 levels of plasma and water samples were quantified via RIA (Licht, McCreery, Barnes, & Pang, 1983).

RNA processing of liver tissue

RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Venlo, NL). Approximately 30 mg of liver from each animal was placed in a 2.0 ml microcentrifuge tube containing one 5 mm stainless steel bead (Qiagen, Venlo, Netherlands) and 600 µl of RLT buffer. The samples were then homogenized with the TissueLyser LT (Qiagen, Venlo, NL) at 50 Hz for five minutes. 300 µl of the homogenate was used for the remaining extraction steps, which may be found in the product handbook under the 'Purification of Total RNA from Animal Tissues,' and the remaining homogenate was stored at -80° C as reserves. On-column DNase digestion of every sample was performed using the RNase-Free DNase Set (Qiagen, Venlo, NL).

The amount of extracted total RNA was quantified using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). 1 µg of total RNA of each sample was reversed transcribed using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Hercules, CA, USA). The resulting cDNA was diluted 1:2 to a working concentration with ultrapure water.

Primer design

Because *X. laevis* are allotetraploid, it was important to use primers specific to the target isoform of each target gene. cDNA was constructed from extracted total RNA and PCR was performed with designed primers for *vtga2* and *esr1*. The resulting amplified DNA fragments were then

sequenced to confirm the identity of the amplicon. All primers were ordered from Integrated DNA Technologies (Coralville, IA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

In addition to our two target genes *vtga2* and *esr1*, two reference genes, *eef1a1* and *rpl8*, were also examined. Reference genes were validated using Biogazelle qbase+ 3.0. Each PCR reaction contained 2.5 µl cDNA, 0.5 µl forward and reverse primers each, 2.5 µl SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories Inc, Hercules, CA, USA), and 0.75 µl ultrapure water. All reactions were done in triplicates.

The primers used:

eef1a1-forward 5′- CAGATTGGTGCTGGATATGC-3′
eef1a1-reverse 5′-ACTGCCTTGATGACTCCTAG-3′
rpl8-forward 5′-ACACGGCATTGATCCTACAG-3′
rpl8-reverse 5′-AGCTCCTTCGGTGTAATGAC-3′
vtga2-foward 5′-AGTGAAAGCAAGATATCTGTG-3′
vtag2-reverse 5′-TTAGGAAGTATGACCTCTGA-3′
esr1-forward 5′-GCATACAAGGACATAATGACTAT-3′
esr1-reverse 5′-TGTTCTTCTTTCTGCCGT-3′

qRT-PCR runs were performed by the StepOnePlus Real-Time PCR System (Applied Biosystems by Life Technologies, Grand Island, NY, USA). Raw fluorescence data was collected from the program and submitted to Real-time PCR Miner, which calculated C_T values for each reaction using a reaction-kinetics-based algorithm (Zhao & Fernald, 2005). The amplification efficiency (E) for each gene was determined by running a standard curve with five 1:5 dilutions of pooled cDNA. The standard curve C_T values were plotted against the log-concentration of each dilution and slope of the resulting trendline was entered into the ThermoFisher Scientific qPCR Efficiency Calculator to calculate E. Expression values for each reaction were then calculated using the following equation:

$$\text{Expression} = \frac{1}{(1+E)^{C_T}}$$

The geometric mean of the reference genes expression values for each sample was calculated and used to normalize the expression of the corresponding target genes. The arithmetic mean of the expression values for the experimental replicates was calculated. The average treatment expression values were then divided by the average control expression values to obtain fold-changes in expression data.

Statistical analysis

Minitab Express 1.5.0 (Minitab Inc., State College, PA, USA) was used to perform all statistical analysis.

RESULTS

Time course experiment

Expression of *vtga2* increased in both populations for all E2 treatment durations except for the baseline time, 0 hours (Figure 1A). Increasing treatment duration, however, was not directly proportional to *vtga2* expression. SD males showed greater fold changes in *vtga2* expression compared to XE males at all treatment durations (Figure 1B). This may be due to XE males expressing higher *vtga2* levels at 0 hours than SD males. Twelve hours of E2 exposure yielded a great difference in *vtga2* expression between the two populations; this duration was chosen for the next experimental phase.

Concentration response experiment

Average plasma E2 levels increased with increasing E2 treatment in both SD and XE populations (Figure 2). Notably, for all treatments, SD males had significantly higher plasma E2 levels than their resistant counterparts. The population fitted means were 34.42 ng/ml plasma E2 for XE and 66.59 ng/ml plasma E2 for SD (Table 1). The "Population" *p*-value was 0.0310, which indicated that XE and SD populations were associated with different plasma E2 levels (Two-way ANOVA: *F*-value = 4.94, *DF* = 1). The fitted means for treatments 0.00 0.03, 0.30, 3.00, 30.00, and 300.00 ng/ml E2 were 0.06, 0.30, 4.04, 19.39, 56.08, and 223.15 ng/ml plasma E2,

respectively (Table 1). The "Treatment" *p*-value was < 0.0001, which indicated that the different E2 treatments were associated with different plasma E2 levels (Two-way ANOVA: *F*-value = 24.81, *DF* = 5). We also examined the interaction between population and treatment; the fitted means for XE-0.00, XE-0.03, XE-0.30, XE-3.00, XE-30.00, and XE-300.00 were 0.00, 0.14, 2.03, 11.16, 33.56, and 159.62 ng/ml plasma E2, respectively, and the fitted means for SD-0.00, SD-0.03, SD-0.30, SD-3.00, SD-30.00, and SD-300.00 were 0.13, 0.46, 6.05, 27.62, 78.60, and 286.68 ng/ml plasma E2, respectively (Table 1). The interaction *p*-value was 0.0963, which indicated that the relationship between treatment concentration and E2 plasma concentration did not depend on population (Two-way ANOVA: *F*-value = 2.00, *DF* = 5).

Individuals' expressions of *vtga2* relative to the reference genes increased in response to E2 treatment for both populations (Figure 3A). However, the increase was not directly proportional to E2 treatment concentration. Before analysis, one extreme outlier from each population was excluded (XE-3.00: 0.3012381598 and SD-3.00: 0.555006888); their values are still displayed and are represented by empty data points. When averaged and compared to control expression levels, SD males showed greater fold change in *vtga2* expression compared to XE males at all treatment concentrations (Figure 3B). Again, this may be due to XE males expressing higher *vtga2* levels at 0.00 ng/ml E2. The population fitted means were 0.013191 for XE *vtga2* expression and 0.005394 for SD *vtga2* expression (Table 2). The "Population" *p*-value was 0.0655, which indicated that SD and XE populations were not associated with different *vtga2* levels (Two-way ANOVA: *F*-value = 3.56, *DF* = 1). The fitted means for treatments 0.00 0.03, 0.30, 3.00, 30.00, and 300.00 ng/ml E2 were 0.001862, 0.000913, 0.000895, 0.009037, 0.005282, and 0.037767 *vtga2* expression, respectively (Table 2). The "Treatment" *p*-value was < 0.0001, which indicated that the different E2 treatments were associated with different *vtga2* expression (Two-way ANOVA: *F*-value = 8.52, *DF* = 5). When looking at the interaction between population and treatment, the fitted means for XE-0.00, XE-0.03, XE-0.30, XE-3.00, XE-30.00, and XE-300.00 were 0.003639, 0.000862,

0.000617, 0.009507, 0.007212, and 0.057310 *vtga2* expression, respectively, and the fitted means for SD-0.00, SD-0.03, SD-0.30, SD-3.00, SD-30.00, and SD-300.00 were 0.000084, 0.000964, 0.001173, 0.008568, 0.003352, and 0.018225 *vtga2* expression, respectively (Table 2). The interaction *p*-value was 0.0468, which indicated that the relationship between treatment concentration and *vtga2* expression did depend on population (Two-way ANOVA: *F*-value = 2.46, *DF* = 5).

Individuals' expression of *esr1* relative to the reference genes increased in response to E2 treatment for both populations (Figure 4A). The response was directly proportional to E2 treatment concentration except for a large decrease in *esr1* expression for both XE and SD at the highest treatment concentration of 300.00 ng/ml E2. When averaged and compared to control expression levels, XE males show greater fold change in *esr1* expression compared to SD males (Figure 4B). The population fitted means were 0.00106419 for XE *esr1* expression and 0.00080108 for SD *esr1* expression (Table 3). The "Population" *p*-value was 0.0227, which indicated that SD and XE populations were associated with different *esr1* levels (Two-way ANOVA: *F*-value = 5.54, *DF* = 1). The fitted means for treatments 0.00 0.03, 0.30, 3.00, 30.00, and 300.00 ng/ml E2 were 0.0002383, 0.0003034, 0.0005704, 0.0014982, 0.0020023, and 0.0009832 *esr1* expression, respectively (Table 3). The "Treatment" *p*-value was < 0.0001, which indicated that the different E2 treatments were associated with different *esr1* expression (Two-way ANOVA: *F*-value = 24.46, *DF* = 5). We also examined the interaction between population and treatment; the fitted means for XE-0.00, XE-0.03, XE-0.30, XE-3.00, XE-30.00, and XE-300.00 were 0.0002001, 0.0003170, 0.0006721, 0.0017721, 0.0023314, and 0.0010924 *esr1* expression, respectively, and the fitted means for SD-0.00, SD-0.03, SD-0.30, SD-3.00, SD-30.00, and SD-300.00 were 0.0002764, 0.0002899, 0.0004687, 0.0012244, 0.0016733, and 0.0008739 *esr1* expression, respectively (Table 3). The interaction *p*-value was 0.4093, which indicated that the relationship between treatment concentration and *esr1* expression did not depend on population (Two-way ANOVA: *F*-value = 1.03, *DF* = 5).

There was a strong, significant correlation between E2 plasma levels and *vtga2* expression (Multiple regression: *adjusted R squared* = 14.51%, *p*-value = 0.0035), while population showed a non-significant correlation with *vtga2* expression (Multiple regression: *adjusted R squared* = 14.51%, *p*-value = 0.0501) (Figure 5). For both populations, there was a decrease in *esr1* expression at the highest treatment concentration of 300.00 ng/ml E2. *esr1* expression up to this concentration was significantly, and strongly correlated to plasma E2 levels (Multiple regression: *adjusted R squared* = 40.66%, *p*-value< 0.0001) and to population (Multiple regression: *adjusted R squared* = 40.66%, *p*-value = 0.0084). However, when including expression values from the highest treatment, there was no significant correlation between E2 plasma levels and *esr1* expression (Multiple regression: *adjusted R squared* = 2.64%, *p*-value = 0.1705) or between population and *esr1* expression (Multiple regression: *adjusted R squared* = 2.64%, *p*-value = 0.1401) (Figure 6).

There was a significant, positive relationship between *vtga2* expression and *esr1* expression for the two populations (Figure 7) (Correlation: Spearman rho = 0.549786, *p*-value< 0.0001).

DISCUSSION

Studies on the effects of E2 on vitellogenin expression have transformed drastically over time (Table 4). Most studies exposed frogs through either a single injection of E2 or conducted *in vitro* studies of tissue or cell culture (Baker & Shapiro, 1978; Green & Tata, 1976; Hayward, Mitchell, & Shapiro, 1980; Palmer & Palmer, 1995; Perlman, Wolffe, Champion, & Tata, 1984; Tata, 1976; van Wyk, Pool, & Leslie, 2003). Experiments were conducting using E2 concentrations ranging from 10^{-11} to 10^{-5} M. Methods of detecting of vitellogenin protein synthesis have improved significantly and thus, earlier studies detected initial vitellogenin induction at 1×10^{-9} M E2 (Wangh & Knowland, 1975), while later studies were able to observe protein induction at concentrations as low as 4.0×10^{-11} M via sandwich ELISAs (Mitsui, Tooi, & Kawahara, 2003, 2007; Nomiyama et al., 2010; Oka et al., 2008). Additionally, more recent

vitellogenin studies qualitatively measured transcription via semiquantitative RT-PCR (Huang, Matthews, Fertuck, & Zacharewski, 2005; Kloas, Lutz, & Einspanier, 1999; Lutz, Blodt, & Kloas, 2005). Since then, the development of qRT-PCR has enabled researchers to more accurately quantify RNA levels in real-time. This current study is the only one to perform *in vivo*, immersion E2 treatment of male *X. laevis*, the exposure route most likely observed in natural conditions, with a wide range of concentrations (including physiologically relevant concentrations), and quantify and compare resulting VTG mRNA and ER mRNA synthesis, simultaneously, via qRT-PCR between animals of two populations.

Immersion in E2 treatment effectively increased plasma E2 levels of male frogs in a concentration-dependent manner. However, when exposed to the same concentration of E2, SD males show higher plasma E2 levels than their XE counterparts. This suggests that SD animals had increased transdermal absorption of the hormone or that XE males were able to more effectively metabolize the hormone from circulation. Both populations showed similar patterns of *vtga2* expression in response to E2, though SD males had higher fold changes in *vtga2* expression compared to XE males. Notably, XE males expressed higher baseline *vtga2* (in both the time course experiment and the concentration response experiment), which led to a smaller fold change in *vtga2* expression in the treatment groups. In both populations, *esr1* expression increased with increasing E2 treatment except for a large decrease at the highest treatment, 300.00 ng/ml. This non-monotonic concentration-response to E2 has been observed for vitellogenin expression in response to E2 treatment previously (Huang et al., 2005). XE animals had higher fold change in *esr1* expression than SD animals, which contradicted the *vtga2* response. This implies that, while E2 directly regulates both genes, the mechanism by which it does so may be different for *esr1* and *vtga2*.

These results indicate that there are inherent differences in susceptibility to the feminizing effects of E2, which can be observed in the population response in sex differentiation of the tadpole gonad (Chapter Two) and *vtga2* expression in the adult liver. Thus, the difference in E2

susceptibility may span tissue types and developmental stages. Although there are several E2 responsive genes, it is apparent that they do not behave equally to E2 exposure across populations or even within the same animal. Animals that responded with high *vtga2* expression did not necessarily show high *esr1* expression or *vice versa*. These data taken together provide evidence that researchers should be aware of these disparities when measuring genetic responses to E2 treatment and that *vtga2* and *esr1* expressions may be indicators of E2 exposure, but not necessarily serve as effective tools to directly measure the degree of *in vivo* exposure.

Future studies should test the effects of ethinyl-estradiol (EE2), a synthetic, more robust form of estrogen, on gene expression between SD and XE animals. Results may indicate whether the population difference in liver gene expression in response to E2 is due to a difference in the animals' ability to metabolize and excrete the hormone from their system. Additional *in vitro* studies using isolated, cultured hepatocytes would allow for a more controlled test environment and address the question of differential E2 metabolism. This would also be an effective method to limit biological variation as cells from a single animal may be used for several treatments and as the control.

ACKNOWLEDGEMENTS

Special thanks to Dr. Michihiko Ito, Dr. Shuuji Mawaribuchi, Mikako Wada, and Kazuko Fujitani at Kitasato University, Sagamihara, Japan for training me on the applied molecular techniques and for their hospitality. Also, thanks for Dr. Sheng Zhao for his training and development of the open source program Real-time PCR Miner. This material is based upon work supported by the National Science Foundation under Grant No. 1209511.

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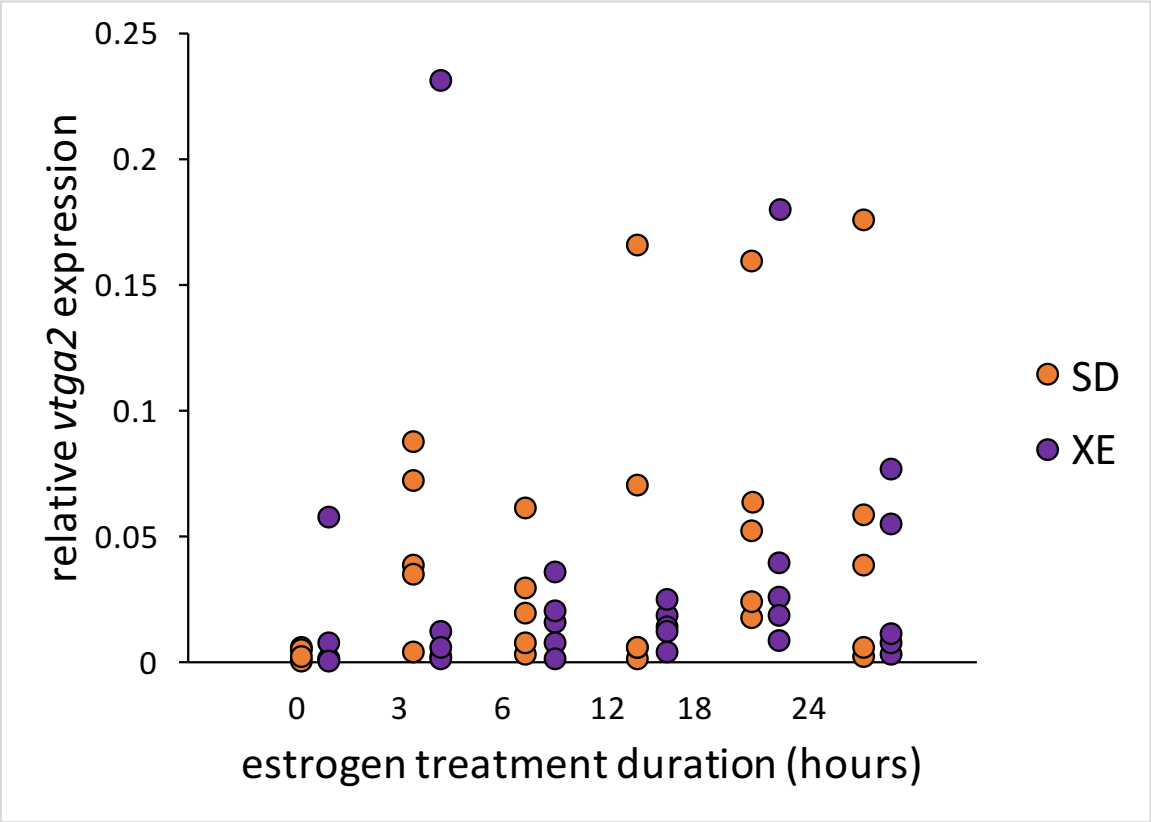

Figure 1A Sample-wise *vtga2* v. E2 treatment duration. Displayed are *vtga2* expression levels resulting from treatment of SD and XE males with 3.00 ng/ml E2 for increasing lengths of time. *n*= 5 for each population at each treatment time.

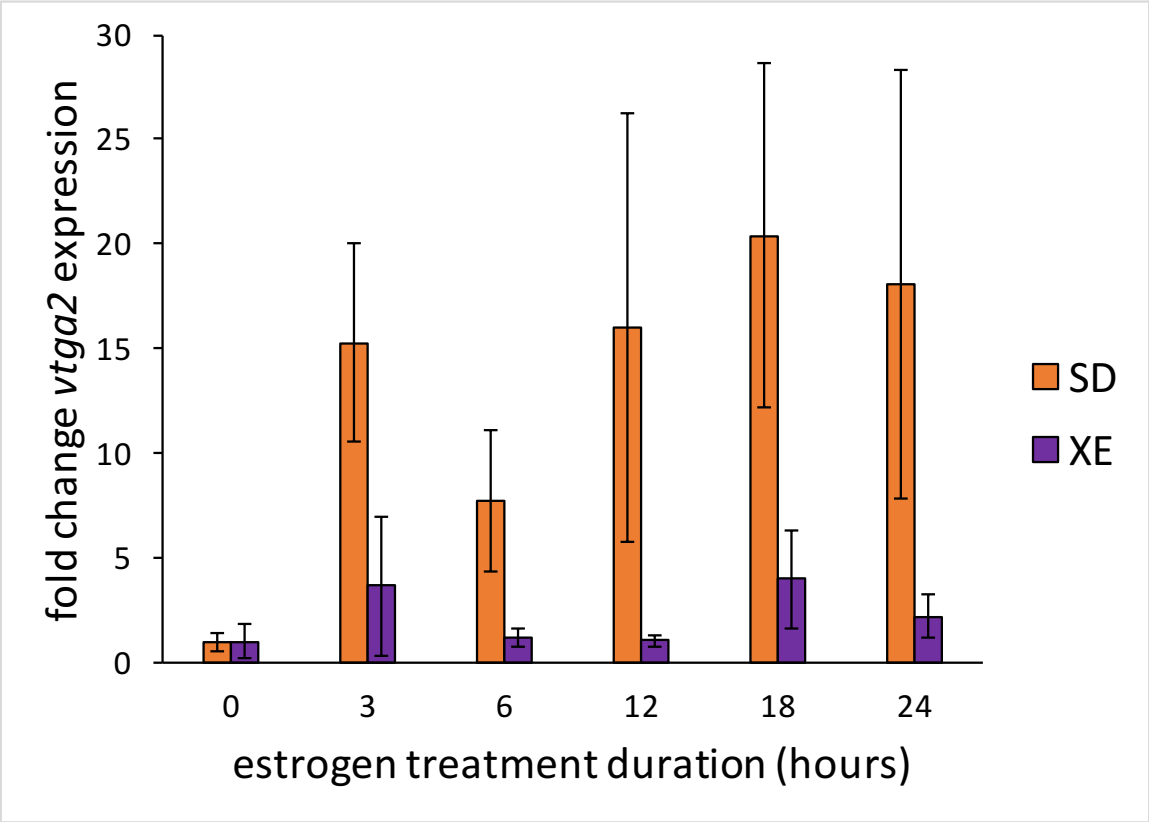

Figure 1B Average fold change *vtga2* v. E2 treatment duration. Displayed are *vtga2* expression levels resulting from treatment of SD and XE males with 3.00 ng/ml E2 for increasing amounts of time. The average *vtga2* expression was calculated for each population at each treatment time (n = 5) and then divided by the baseline expression of the corresponding population at t = 0 hours (n = 5).

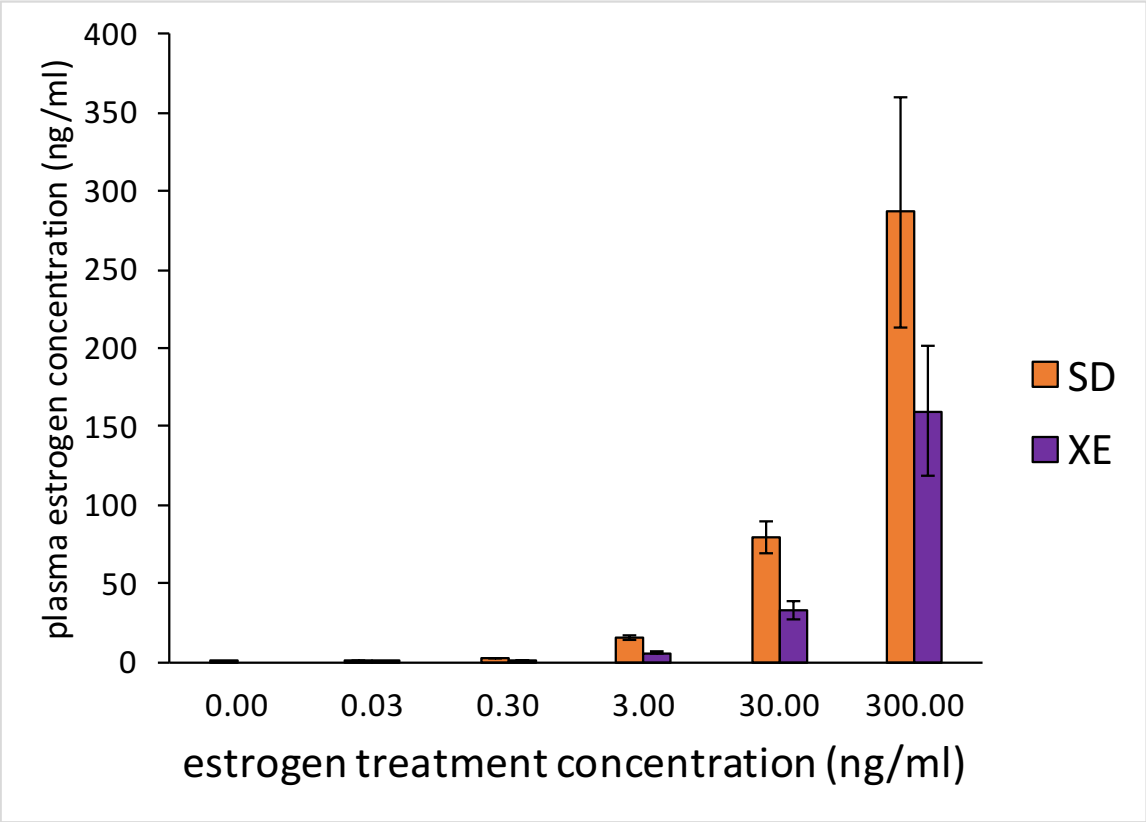

Figure 2 Average E2 plasma concentration v. E2 treatment. SD and XE animals were treated with increasing E2 concentrations for 12 hours (n = 5 for each population at each treatment concentration). Their plasma was extracted and E2 concentration measured via RIA. The p-value for "Population" was 0.0310, which indicated that SD and XE populations were associated with different plasma E2 levels (Two-way ANOVA: F-value = 4.94, DF = 1). The p-value for "Treatment" was < 0.0001, which indicated that the different E2 treatments were associated with different plasma E2 levels (Two-way ANOVA: F-value = 24.81, DF = 5). However, the interaction, "Population*Treatment," p-value was 0.0963, which indicated that the relationship between treatment concentration and E2 plasma concentration did not depend on population (Two-way ANOVA: F-value = 2.00, DF = 5).

Means

Term	Fitted Mean	SE Mean
Population		
SD	66.59	10.23
XE	34.42	10.23
Treatment		
0.00	0.06	17.60
0.03	0.30	17.60
0.30	4.04	16.07
3.00	19.39	17.60
30.00	56.08	19.68
300.00	223.15	17.60
Population*Treatment		
SD 0.00	0.13	24.90
SD 0.03	0.46	24.90
SD 0.30	6.05	22.73
SD 3.00	27.62	24.90
SD 30.00	78.60	27.83
SD 300.00	286.68	24.90
XE 0.00	0.00	24.90
XE 0.03	0.14	24.90
XE 0.30	2.03	22.73
XE 3.00	11.16	24.90
XE 30.00	33.56	27.83
XE 300.00	159.62	24.90

Table 1 ANOVA: E2 plasma concentration of E2-treated males. SD and XE animals were treated with increasing E2 concentrations for 12 hours (n = 5 for each population at each treatment concentration). Their plasma was extracted and E2 concentration measured via RIA. The p-value for "Population" was 0.0310, which indicated that SD and XE populations were associated with different plasma E2 levels (Two-way ANOVA: F-value = 4.94, DF = 1). The p-value for "Treatment" was < 0.0001, which indicated that the different E2 treatments were associated with different plasma E2 levels (Two-way ANOVA: F-value = 24.81, DF = 5). However, the interaction, "Population*Treatment," p-value was 0.0963, which indicated that the relationship between treatment concentration and E2 plasma concentration did not depend on population (Two-way ANOVA: F-value = 2.00, DF = 5).

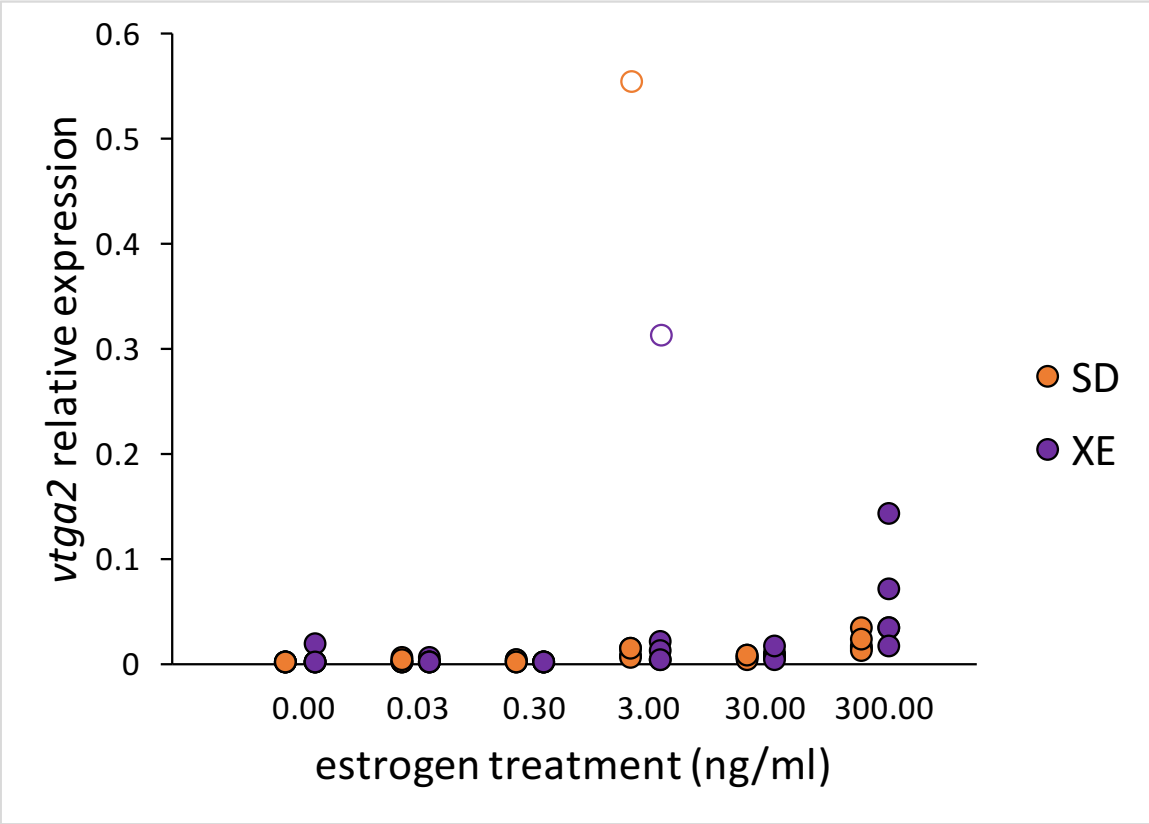

Figure 3A Sample-wise *vtga2* v. E2 treatment. Displayed are *vtga2* expression levels resulting from treatment of SD and XE males with increasing E2 concentrations for 12 hours. Empty data points represent outliers that were excluded from statistical analysis. n = 5 for each population at each treatment concentration.

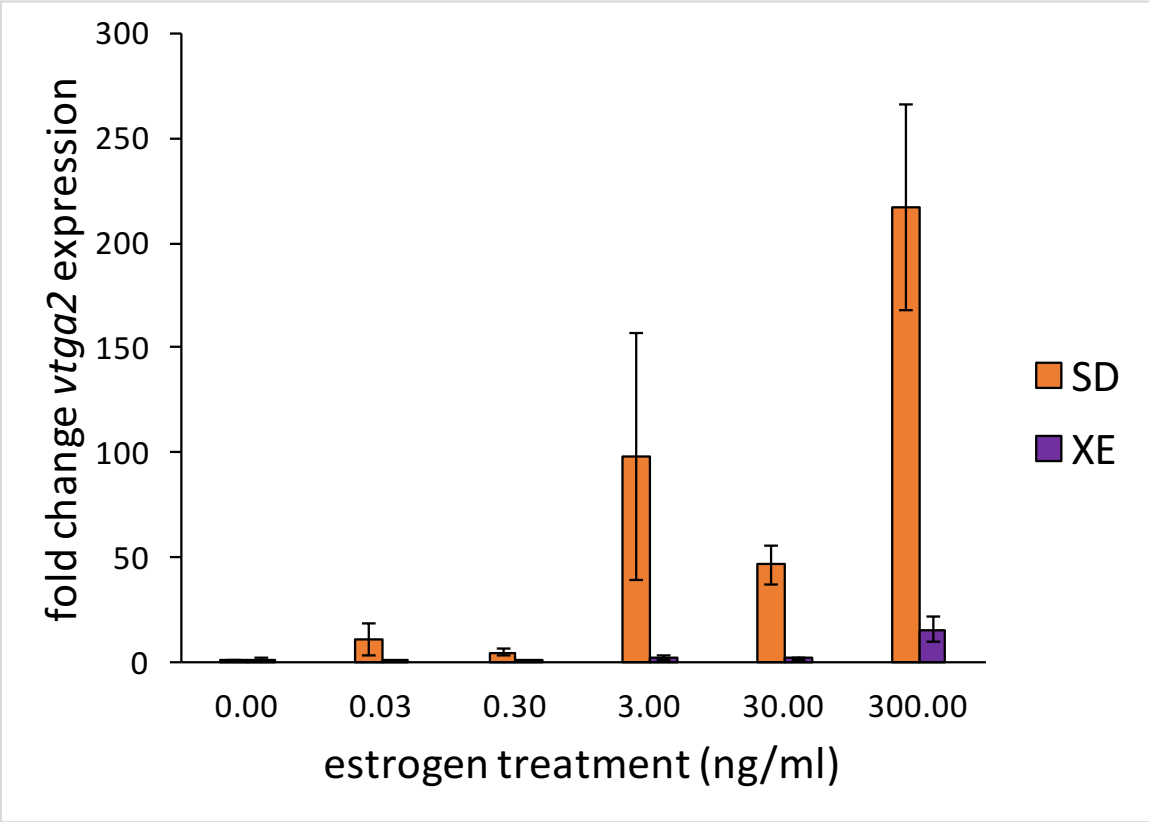

Figure 3B Average fold change in *vtga2* v. E2 treatment. Displayed are *vtga2* expression levels resulting from treatment of SD and XE males with increasing E2 concentrations for 12 hours. The average *vtga2* expression was calculated for each population at each treatment concentration (n = 5) and then divided by the control expression of the corresponding population at 0.00 ng/ml E2. The "Population" p-value was 0.0655, which indicated that SD and XE populations were not associated with different *vtga2* levels (Two-way ANOVA: F-value = 3.56, DF = 1). The "Treatment" p-value was < 0.0001, which indicated that the different E2 treatments were associated with different *vtga2* expression (Two-way ANOVA: F-value = 8.52, DF = 5). The interaction p-value was 0.0468, which indicated that the relationship between treatment concentration and *vtga2* expression did depend on population (Two-way ANOVA: F-value = 2.46, DF = 5).

Means

Term	Fitted Mean	SE Mean
Population		
SD	0.005394	0.002922
XE	0.013191	0.002922
Treatment		
0.00	0.001862	0.004925
0.03	0.000913	0.004925
0.30	0.000895	0.004496
3.00	0.009037	0.005507
30.00	0.005282	0.005507
300.00	0.037767	0.004925
Population*Treatment		
SD 0.00	0.000084	0.006966
SD 0.03	0.000964	0.006966
SD 0.30	0.001173	0.006359
SD 3.00	0.008568	0.007788
SD 30.00	0.003352	0.007788
SD 300.00	0.018225	0.006966
XE 0.00	0.003639	0.006966
XE 0.03	0.000862	0.006966
XE 0.30	0.000617	0.006359
XE 3.00	0.009507	0.007788
XE 30.00	0.007212	0.007788
XE 300.00	0.057310	0.006966

Table 2 ANOVA: *vtga2* expression of E2-treated males. SD and XE animals were treated with increasing E2 concentration for 12 hours (n = 5 for each population at each treatment concentration). The livers were dissected out, RNA was extracted, and *vtga2* expression quantified via qRT-PCR. The "Population" p-value was 0.0655, which indicated that SD and XE populations were not associated with different *vtga2* levels (Two-way ANOVA: F-value = 3.56, DF = 1). The "Treatment" p-value was < 0.0001, which indicated that the different E2 treatments were associated with different *vtga2* expression (Two-way ANOVA: F-value = 8.52, DF = 5). The interaction p-value was 0.0468, which indicated that the relationship between treatment concentration and *vtga2* expression did depend on population (Two-way ANOVA: F-value = 2.46, DF = 5).

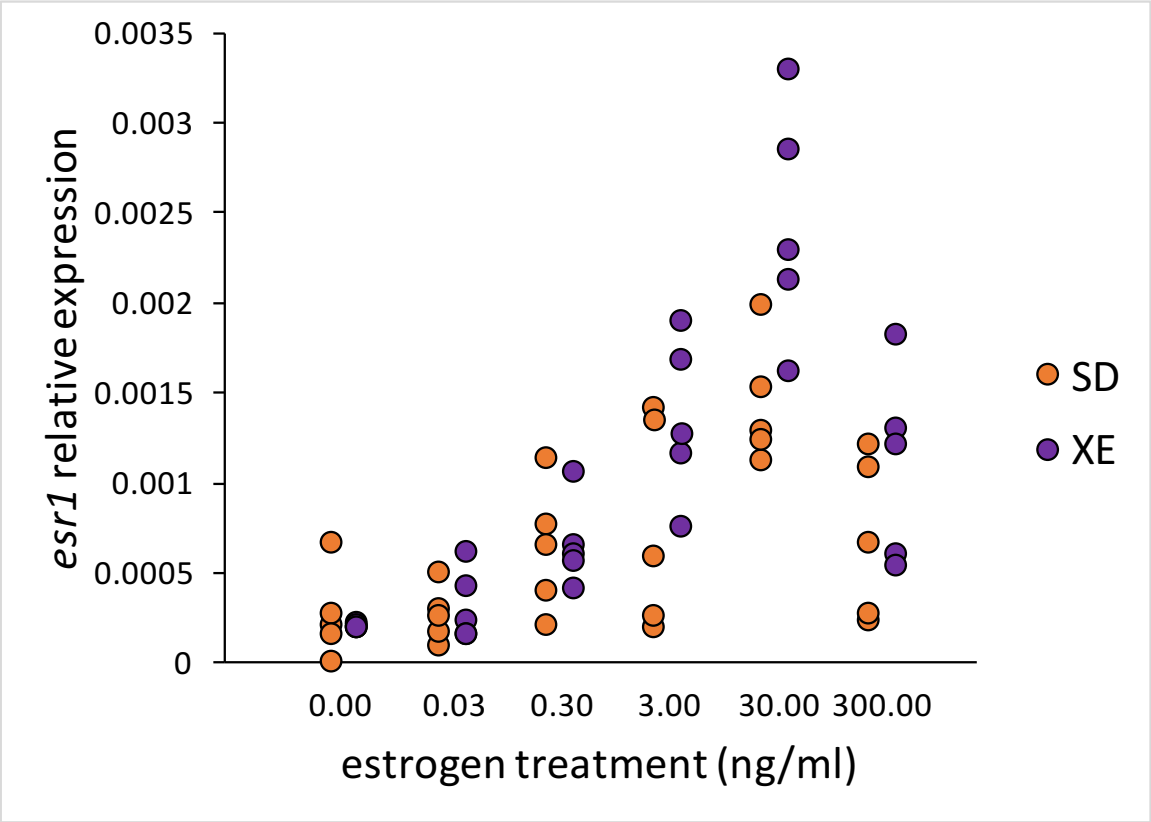

Figure 4A Sample-wise *esr1* v. E2 treatment. Displayed *esr1* expression levels resulting from treatment of SD and XE males with increasing E2 concentrations for 12 hours. n = 5 for each population at each treatment concentration

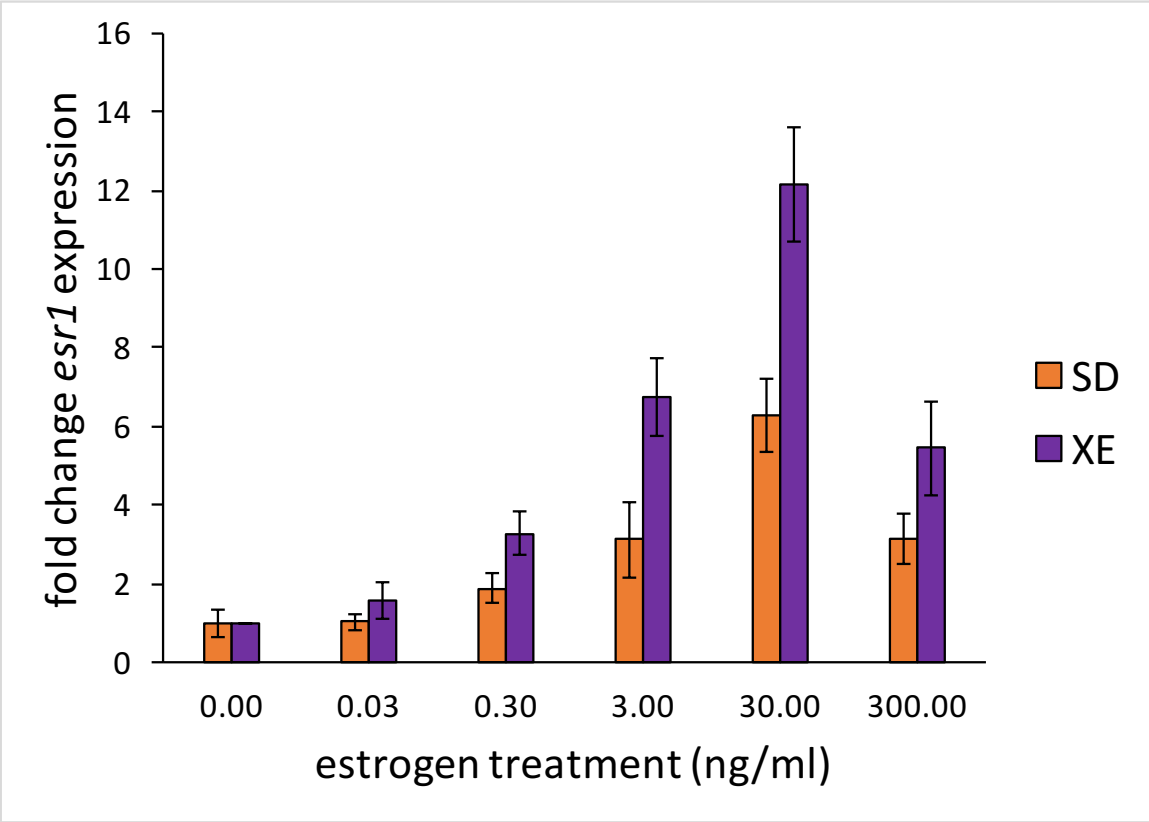

Figure 4B Average fold change in *esr1* v. E2 treatment. *esr1* expression levels resulting from treatment of SD and XE males with increasing E2 concentrations for 12 hours. The average *esr1* expression was calculated for each population at each treatment concentration ($n = 5$) and then divided by the control expression of the corresponding population at 0.00 ng/ml E2. The "Population" p-value was 0.0227, which indicated that SD and XE populations were associated with different *esr1* levels (Two-way ANOVA: F-value = 5.54, $DF = 1$). The "Treatment" p-value was < 0.0001, which indicated that the different E2 treatments were associated with different *esr1* expression (Two-way ANOVA: F-value = 24.46, $DF = 5$). The interaction p-value was 0.4093, which indicated that the relationship between treatment concentration and *esr1* expression did not depend on population (Two-way ANOVA: F-value = 1.03, $DF = 5$).

Means

Term	Fitted Mean	SE Mean
Population		
SD	0.00080108	0.00007902
XE	0.00106419	0.00007902
Treatment		
0.00	0.0002383	0.0001359
0.03	0.0003034	0.0001359
0.30	0.0005704	0.0001241
3.00	0.0014982	0.0001359
30.00	0.0020023	0.0001520
300.00	0.0009832	0.0001359
Population*Treatment		
SD 0.00	0.0002764	0.0001922
SD 0.03	0.0002899	0.0001922
SD 0.30	0.0004687	0.0001755
SD 3.00	0.0012244	0.0001922
SD 30.00	0.0016733	0.0002149
SD 300.00	0.0008739	0.0001922
XE 0.00	0.0002001	0.0001922
XE 0.03	0.0003170	0.0001922
XE 0.30	0.0006721	0.0001755
XE 3.00	0.0017721	0.0001922
XE 30.00	0.0023314	0.0002149
XE 300.00	0.0010924	0.0001922

Table 3 ANOVA: *esr1* expression of E2-treated males. SD and XE animals were treated with increasing E2 concentrations for 12 hours (n = 5 for each population at each treatment concentration). The livers were dissected out, RNA was extracted, and *esr1* expression quantified via qRT-PCR. The "Population" p-value was 0.0227, which indicated that SD and XE populations were associated with different *esr1* levels (Two-way ANOVA: F-value = 5.54, DF = 1). The "Treatment" p-value was < 0.0001, which indicated that the different E2 treatments were associated with different *esr1* expression (Two-way ANOVA: F-value = 24.46, DF = 5). The interaction p-value was 0.4093, which indicated that the relationship between treatment concentration and *esr1* expression did not depend on population (Two-way ANOVA: F-value = 1.03, DF = 5).

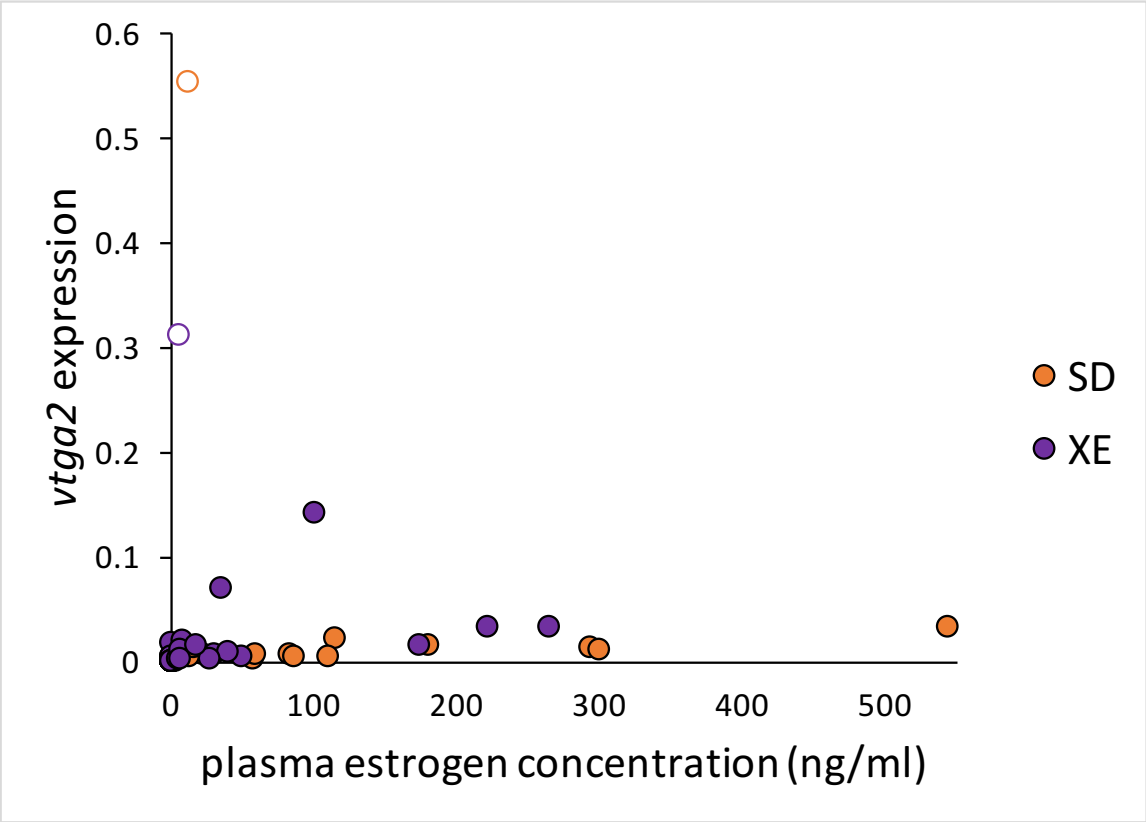

Figure 5 Sample-wise *vtga2* v. E2 plasma concentration. Displayed are the *vtga2* expression levels for each individual plotted against their own E2 plasma concentration. n = 5 for each population at each treatment concentration. Before analysis, one extreme outlier from each population was excluded; their values are still displayed and are represented by empty data points. There was a strong, significant correlation between E2 plasma levels and *vtga2* expression (Multiple regression: *adjusted R squared* = 14.51%, p-value = 0.0035), while population showed a non-significant correlation with *vtga2* expression (Multiple regression: *adjusted R squared* = 14.51%, p-value = 0.0501).

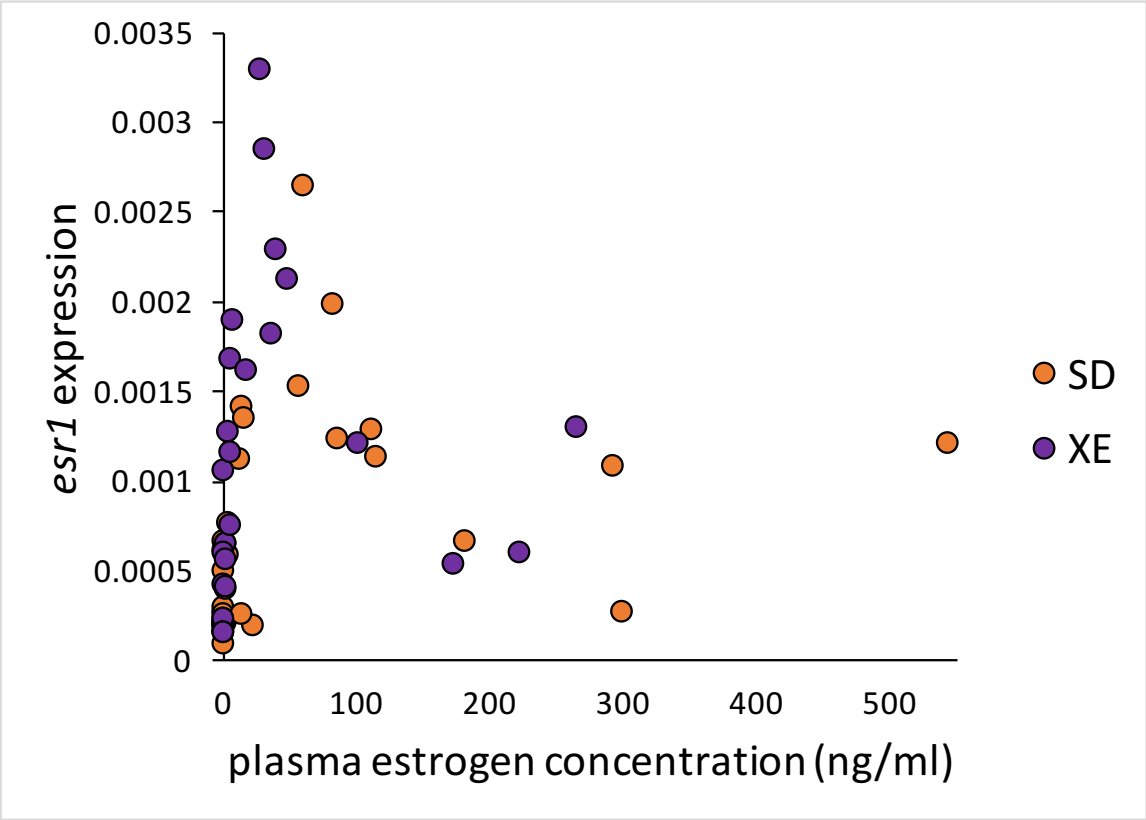

Figure 6 Sample-wise *esr1* v. E2 plasma concentration. Displayed are the *esr1* expression levels for each individual plotted against their own E2 plasma concentration. *n* = 5 for each population at each treatment concentration. For both populations, there was a decrease in *esr1* expression at the highest treatment concentration of 300.00 ng/ml E2. *esr1* expression up to this concentration was significantly, and strongly correlated to plasma E2 levels (Multiple regression: *adjusted R squared* = 40.66%, *p*-value<0.0001) and to population (Multiple regression: *adjusted R squared* = 40.66%, *p*-value = 0.0084). However, when including expression values from the highest treatment, there was no significant correlation between E2 plasma levels and *esr1* expression (Multiple regression: *adjusted R squared* = 2.64%, *p*-value = 0.1705) nor between population and *esr1* expression (Multiple regression: *adjusted R squared* = 2.64%, *p*-value = 0.1401).

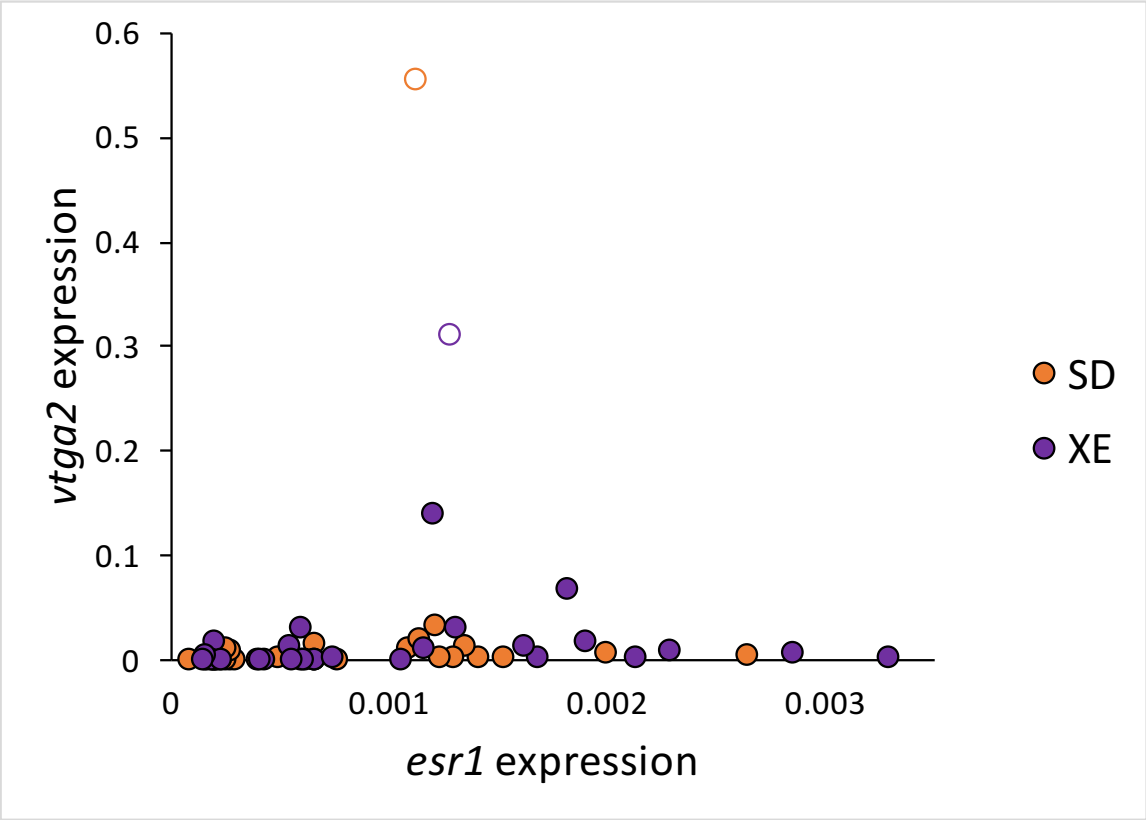

Figure 7 Sample-wise *vtga2* v. sample-wise *esr1*. Displayed are the *esr1* expression levels for each individual plotted against their own *esr1* expression. $n = 5$ for each population at each treatment concentration. There was a positive relationship between the two variables (Correlation: Spearman rho = 0.549786, p-value < 0.0001).

Year	Author(s)	Exposure(s)	Treatment(s)	Method(s)	Biomolecule(s) detected	Results
1975	Wangh & Knowland	*In vitro* – tissue culture	1.84 x 10^{-10} M – 1.84 x 10^{-6} M E2 for 8 days	[^{35}S]Methionine protein labeling & gel electrophoresis	Protein	Induction of VTG synthesis in males starts at 1.84 X 10^{-9} M E2 (similar to normal E2 plasma levels of female vertebrates)
1976	Green & Tata	*In vitro* – tissue culture	10^{-10} M – 10^{-6} M E2 for 8 days	[^{35}S]Methionine protein labeling & gel electrophoresis	Protein	Significant VTG induction in males with 10^{-10} M E2; maximal VTG synthesis with 10^{-8} M E2 or greater; VTG first detectable on day 4 of culture and reaches maximum on day 8
1977	Baker & Shapiro	*In vivo* – injection	0.4 mg E2/10 g body weight (~ 2 mg)	Indirect immunoprecipitation	mRNA	VTG mRNA sequences clearly detected 4.5 hours after E2 injection of males; rates of initial mRNA synthesis exhibit the most significant variation among individuals due to variations in rate of E2 uptake and consequent variation in time required to achieve a saturating level of E2 in the liver
1979	Westley & Knowland	*In vivo* – injection	1 mg E2	Radioreceptor assay	Protein	Liver nuclear ER rises within 6 hours of injection in males, reaching maximal levels within 12 hours; increase in nuclear ER due to *de novo* synthesis; VTG synthesis detected only after 12 hours
1980	Hayward et al.	*In vivo* - injection	2 mg E2	Radioreceptor assay	Protein	E2 injection in male Xenopus induces both VTG synthesis and nuclear ER; withdrawal from E2 for 70-75 days showed nuclear ER level returning to levels seen in unstimulated cells while the cytoplasmic ER increased 8- to 12-fold; with E2 restimulation, VTG levels increased more so than in primary stimulation, but nuclear ER levels remain the same; maximal level of VTG synthesis not necessarily depending on nuclear ER
1981	Searle & Tata	*In vitro* – cell culture	1) 10^{-6} M E2 2) 10^{-9} M – 10^{-5} M E2 for 5 or 8 days	Disc hybridization	mRNA	1) VTG mRNA accumulation detected 2 hours after exposure of males; maximum rate of accumulation seen after 5-6 hours 2) Small but significant response at 10^{-9} M E2; E2 half-life of 1-1.5 hours in culture

Year	Author(s)	Exposure(s)	Treatment(s)	Method(s)	Biomolecule(s) detected	Results
1983	Wright et al.	*In vivo* – injection	1 mg E2	1) Radioreceptor assay or fluorescamine 2) Radioimmunoassay	1) Protein 2) Hormone	Control male liver [E2] = 0.18 nM with ~100 E2 binding site/nucleus; control female liver [E2] =3.5 nM with ~440 sites/nucleus; 6 hours after E2 injection, rapid rise of ER in male livers; amount of ER is variable in physiological range of [E2]; relatively small increase in VTG mRNA induced by physiological [E2] is due to pre-existing ER and larger increases induced by very high [E2] depends on newly-synthesized receptor; alternatively, requirement for very high [E2] for highest levels of VTG gene expression reflects rapid E2 metabolism
1984	Perlman et al.	*In vitro* – cell culture	10^{-6} M E2 every 4 hours for 12 hours and/or 10 ug/ml cycloheximide and/or 10^{-6} M tamoxifen	1) Receptor assay 2) Disc hybridization	1) Protein 2) mRNA	Transcription of VTG genes in naive male cells was clearly measurable 1 hour after exposure to E2; rate of VTG transcription is directly proportional to ER number; cycloheximide-treated cells exhibited a short burst of transcription (most likely produced by pre-existing ER) followed by an abolition of E2-induced transcription; prior exposure to tamoxifen led to a gradual decrease in ER and failed to increase the rate of transcription of VTG genes
1987	Riegel et al.	*In vivo* - injection	0.5 mg E2 and/or 1.0 mg 4-hydroxytamoxifen	1) Receptor exchange assay 2) Nuclear transcription run-on assay	1) Protein 2) RNA	4-hydroxytamoxifen was able to completely block E2-induced VTG synthesis in male livers, but only partially decrease ER synthesis
1995	Palmer & Palmer	*In vivo* – injection	1 ug E2/g body weight, daily for 7 days	ELISA and 1-D-SDS-PAGE	Protein	Significantly increased VTG production in E2-treated males
1999	Kloas et al.	*In vitro* – cell culture	1) Time course with 10^{-6} M E2	Semiquantitative RT-PCR	mRNA	1) In controls, VTG mRNA broke down to very low levels in 2 days while E2 treatment kept mRNA elevated to levels at which the cell culture started with

Year	Author(s)	Exposure(s)	Treatment(s)	Method(s)	Biomolecule(s) detected	Results
			2) 10^{-10} M – 10^{-5} M E2 for 36 hours			2) E2 caused an increase of VTG mRNA starting at 10^{-9} M E2 and reached saturation 10^{-6} M E2
2002	van Wyk et al.	*In vivo* - injection	10 ug E2/g body weight/week for 28 days	ELISA	Protein and hormone	Decreased plasma testosterone and increased VTG production in E2-treated males
2003	Mitsui et al.	1) *In vivo* – cell culture 2) *In vivo* - immersion	1) 10^{-10} – 10^{-7} M E2 for 6 days 2) 10^{-10} – 10^{-6} M E2 for 7 days	Sandwich ELISA	Protein	1) VTG induction was detected after exposure to at least 10^{-9} M E2; variability in VTG response among different cell culture preps 2) Younger male frogs showed lower levels of VTG induction; older male frogs, showed high levels of VTG induction, although the range of values was fairly large
2005	Lutz et al.	*In vitro* – cell culture	1) Time course with 10^{-8} M and 10^{-6} M E2 2) 10^{-10} M – 10^{-6} M E2 for 36 hours	1) Radioreceptor assay 2) Semiquantitative RT-PCR	1) Protein 2) mRNA	1) 10^{-6} M E2 caused faster drop of free ER within the first hour and was raised again to similar levels as controls after 12 hours 2) ER mRNA drops in controls within the first 12 hours; E2 treatment maintains ER mRNA over the entire time course until 72 hours; E2 significantly induced ER mRNA at 10^{-9} M and reached saturation level at 10^{-6} M
2005	Huang	*In vivo* Injection	0.15 mg/kg – 15 mg/kg E2 cumulative	Semiquantitative RT-PCR	mRNA	VTG mRNA increased starting at 0.15 mg/kg E2 with maximal increase at 3 mg/kg E2, but decreased at 15 mg/kg
2007	Mitsui et al.	*In vivo* – cell culture	10^{-11} M – 10^{-6} M E2 for 6 days	Sandwich ELISA	Protein	Induction of VTG was first observed at 4 x 10^{-11} M E2
2008	Oka et al.	1) *In vivo* – immersion 2) *In vitro* – cell culture	1) 10^{-9} M E2 from stage 49-66 2) 10^{-11} – 10^{-8} M E2 for 6 days	Sandwich ELISA	Protein	1) VTG was induced in the all-male ZZ froglets exposed to E2 2) Concentration-dependent increase of VTG in culture media
2010	Nomiyama et al.	*In vitro* – cell culture	1.0×10^{-8} – 4.1×10^{-12} M E2	Sandwich ELISA	Protein	3.7 x 10^{-11} M E2 induced 1.5 ng/mL VTG

Table 4 Estradiol-induced liver gene expression literature review. Above are summaries of notable papers that examined estradiol- (E2) induced vitellogenin (VTG) and estrogen receptor (ER) expression in the *X. laevis* liver. This current study is the only one to perform *in vivo* submersion E2 treatment (3.00 ng/ml E2 = 11 nM E2 = 1.1 x 10^{-8} M E2) of male *X. laevis*, the exposure route most likely observed in natural conditions, and simultaneously quantified and compared resulting VTG mRNA and ER mRNA synthesis via qRT-PCR between animals of two populations.

Chapter Four

RNA-Seq Confirms Population Gene Expression Differences in the Developing Gonads

ABSTRACT

Xenopus laevis is a female heterogametic species, females are ZW and males are ZZ. *DM-W*, located on the W chromosome, is the female sex determining gene, while *DMRT1* (*dmrt1.S*) is autosomal and important in testes development. Expression of *DM-W* leads to competitive, antagonistic binding to *DMRT1*-activated cis-regulatory regions of male-specific genes, resulting in suppression of testicular differentiation. Simultaneously, *DM-W* increases the expression of aromatase (*cyp19a1.L*), the enzyme responsible for estrogen synthesis. Ultimately, the increase in estrogen production during sex differentiation leads to ovarian development.

The goal of this study was to elucidate the mechanism by which exogenous estradiol (E2) feminizes undifferentiated gonads in ZZ tadpoles in the absence of *DM-W*. In Chapters Two and Three, we have characterized large disparities in E2 susceptibility among populations of *X. laevis* frogs. E2-susceptible tadpoles (SD) had higher male-to-female sex reversal rates than their E2-resistant counterparts (XE) and adult SD and XE males show different degrees of vitellogenin upregulation at any given E2 concentration. Using RNA sequencing (RNA-Seq), we examined universal gene expression in the developing gonad and how it varied between XE and SD tadpoles in response to 3.00 ng/ml estradiol (E2) exposure. When comparing control SD and XE, the expressions of *ahr.S* and *hsd17b7.S* were greater in SD males than in XE males, while the expression of *cyp2c8.2.L* was less in SD males than in XE males. In SD-E2 males, *cyp19a1.L* expression was greater, while *dmrt1.s* expression was lower than in SD-CON. In XE-E2 males, *hsd17b7.S* expression was increased compared to XE-CON males. In E2-treated animals from both populations, *hsd17b7.S* expression was greater in XE males than in SD males. From these findings, we propose a mechanism underlying susceptibility to feminization, which involves the suppression of masculinizing genes, upregulation of feminizing genes, and differential E2 production and metabolism. Additionally, future quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments must be conducted in E2-treated gonads

of animals from both populations to confirm observed changes in expression of *ahr.S*, *hsd17b7.S*, *cyp2c8.2.L*, *dmrt1.S*, and *cyp19a1.L*.

INTRODUCTION

Although most amphibians lack discernable sex chromosomes, all amphibians display genetic sex determination (Eggert, 2004). Sex chromosomes have been identified in *Xenopus laevis*, where females are heterogametic (ZW) and males are homogametic (ZZ). *DM-W*, a W-linked gene and paralog of *DMRT1* (*dmrt1.S*), is the female sex determining gene in this species (Yoshimoto & Ito, 2011). *DM-W* is transiently and specifically upregulated in the ZW gonad during sex determination at developmental stage 50 (Yoshimoto et al., 2010). Ectopic expression of *DMRT1* induced primary testicular development in some ZW individuals. The expression of *DM-W* results in competitive, antagonistic binding to *DMRT1*-activated cis-regulatory regions of male-specific genes, resulting in the suppression of testicular differentiation (Yoshimoto et al., 2010). Simultaneously, *DM-W* increases the expression of *cyp19a1.L*, which codes for aromatase, the enzyme responsible for estrogen synthesis (Okada et al., 2009). Ultimately, the increase in estrogen production during sex differentiation leads to ovarian development (Miyashita, Shimizu, Osanai, & Miyata, 2000).

Changes in temperature, physical manipulations, and exposure to endocrine-disrupting chemicals may alter sex differentiation in amphibians resulting in populations with skewed sex ratios (Hayes et al., 2006). Researchers, therefore, can readily study the role of specific environmental factors on development by manipulating the rearing conditions of developing tadpoles. In particular, *X.laevis* ZZ tadpoles exposed to E2 during a critical window during sex determination may become sex-reversed and mature into sexually functional ZZ females (Villalpando & Merchant-Larios, 1990). Conversely, exposure to an aromatase or E2 inhibitor can result in a to male phenotype (Miyata & Kubo, 2000). The ability to breed ZZ males and ZZ females and obtain all-ZZ male tadpoles

allow for researchers to perform important sex development experiments (Oka et al., 2006).

We aim to elucidate the mechanism by which exogenous E2 feminizes the undifferentiated gonad in males (in the absence of *DM-W*). Whole transcriptome shotgun sequencing, also known as RNA-Seq, offers the ability to discover new genes and transcripts and quantify transcript expression at a given moment in time. Using this technique, we examined universal gene expression in specific tissues and how it varied between populations and in response to E2 treatment. We predicted that SD males would be more susceptible to feminization and thus, will have increased expression of female-associated genes and decreased expression of male-associated genes compared to XE males.

MATERIALS and METHODS

Animals

ZZ tadpoles from E2-susceptible (SD) and E2-resistant (XE) populations were treated with either 0.00 or 3.00 ng/ml E2 beginning at 10 days posthatch (dph) until sex differentiation (NF stages 52 and 53). Tadpoles were bred from four SD parent pairs and two XE parent pairs. Animals were then euthanized and the gonad-kidney complexes were dissected out, preserved in RNAlater (Qiagen, Venlo, NL), and stored at -80° C.

RNA processing

RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Venlo, NL). Tissue from 10 animals were pooled for each group and placed in 2.0 ml microcentrifuge tubes containing one 5 mm stainless steel bead (Qiagen, Venlo, NL) and 300 ml of RLT buffer each. Samples were homogenized with a TissueLyser LT (Qiagen, Venlo, NL) at 50 Hz for five minutes. The remaining extraction steps may be found in the product handbook under the 'Purification of Total RNA from Animal Tissues.' The extracted RNA was then treated with DNase using the RNase-Free DNase Set (Qiagen, Venlo, NL). Total RNA was sent to the California Institute for

Quantitative Biosciences's Functional Genomics Laboratory (FGL) for sample quality check using the Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) fragment analysis. All samples had two clear peaks at 18S and 28S and concentrations were verified. All RNA integrity values were 9.3 and above.

cDNA library preparation and RNA-Seq

A cDNA library was constructed by the FGL and the prepared library's quality was checked via Agilent 2100 Bioanalyzer fragment analysis. RNA-Seq was performed by the Vincent J. Coates Genomic Sequencing Laboratory at the University of California, Berkeley using the HiSeq 2000, Illumina platform (San Diego, CA, USA). Sequence outputs were in the form of Illumina `fastq` files.

Sequence quality assessment

FASTQC was used to check the quality of the raw sequence data. For each `fastq` file, the program reported basic statistics, per base sequence quality, per sequence quality scores, per base sequence content, per base GC content, per sequence GC content, per base N content, sequence length distribution, duplicate sequences, overrepresented sequences, and overrepresented Kmers. Typically, quality scores greater than 20 and means of 50% of GC content per read are acceptable.

Filtering reads

The concatenated sequences files were then filtered and trimmed using Trimmomatic (Bolger, Lohse, & Usadel, 2014). We used `ILLUMINACLIP` to excise Illumina TruSeq3 single-ended adapters from the reads. `LEADING` and `TRAILING` cut bases with quality score lowering than 3 at the start and end of a read, respectively. `SLIDINGWINDOW` averaged the quality of four bases and cuts when the average quality per base drops below 15. `MINLEN` specified that reads must be at minimum 36 bases. The outputs were `trim.fastq` files.

```
default: stats
```

```
# Base directory
base_dir = /Volumes/HayesLabHardDrive

# Source directory
fq_dir = $(base_dir)/THXL
fq_files = $(wildcard $(fq_dir)/*.fastq.gz)
basenames_ext = $(foreach x,$(fq_files),$(basename $(basename
$(notdir $(x)))))
basenames = $(shell echo $(basenames_ext) | xargs -n1 echo | sed
-e "s/_[0-9]\\{1,3\\}$$//g" | sort -u)

# Concatenated FQ directory and files
fqc_dir = $(base_dir)/fastq_cat
fqc_files = $(foreach x,$(basenames),$(fqc_dir)/$(x).fastq.gz)

# Trimmed FQ directory and files
fqct_dir = $(base_dir)/fastq_cat_trim
fqctgz_files = $(foreach
x,$(basenames),$(fqct_dir)/$(x)_trim.fastq.gz)
fqct_files = $(foreach
x,$(basenames),$(fqct_dir)/$(x)_trim.fastq)
```

Mapping reads and obtaining gene counts

The annotated transcriptome for *X. laevis* (version 9.1) was downloaded from Xenbase (http://www.xenbase.org/, RRID:SCR_003280) (Karpinka et al., 2015) and an index reference was built using `Bowtie2` in RSEM (Langmead & Salzberg, 2012; Li & Dewey, 2011). `trim.fastq` files were then aligned to the downloaded reference transcriptome. The outputs were `gene.results` files. A count matrix was created using Trinity (Grabherr et al., 2011). The output was one file containing all gene identifiers and counts called `genes.counts.matrix`. Finally, official Xenbase gene symbols were mapped to their respective NCBI GenInfo Identifier (GI number). The output was `genes.counts.symbols`. Any unannotated transcripts were labeled as 'ND' (not determined).

```
# Download transcriptome
# Build index reference with rsem and bowtie2
wget
ftp://ftp.xenbase.org/pub/Genomics/Sequences/xlaevisMRNA.fasta
rsem-prepare-reference --bowtie2 -p 2 xlaevisMRNA.fasta XL9

# Calculate expression values
```

```
rsem-calculate-expression -p 8 --bowtie2 /media/hayes/genome/XL9
THXL1A
rsem-calculate-expression -p 8 --bowtie2 /media/hayes/genome/XL9
THXL1B
rsem-calculate-expression -p 8 --bowtie2 /media/hayes/genome/XL9
THXL1C
rsem-calculate-expression -p 8 --bowtie2 /media/hayes/genome/XL9
THXL1D
rsem-calculate-expression -p 8 --bowtie2 /media/hayes/genome/XL9
THXL1E
rsem-calculate-expression -p 8 --bowtie2 /media/hayes/genome/XL9
THXL1F
rsem-calculate-expression -p 8 --bowtie2 /media/hayes/genome/XL9
THXL07
rsem-calculate-expression -p 8 --bowtie2 /media/hayes/genome/XL9
THXL08
rsem-calculate-expression -p 8 --bowtie2 /media/hayes/genome/XL9
THXL09
rsem-calculate-expression -p 8 --bowtie2 /media/hayes/genome/XL9
THXL10
rsem-calculate-expression -p 8 --bowtie2 /media/hayes/genome/XL9
THXL11
rsem-calculate-expression -p 8 --bowtie2 /media/hayes/genome/XL9
THXL12

# Create count matrix
~/bin/trinityrnaseq-2.1.0/util/abundance_estimates_to_matrix.pl
--est_method RSEM /media/hayes/rsem_out/THXL1A.genes.results
/media/hayes/rsem_out/THXL1B.genes.results
/media/hayes/rsem_out/THXL1C.genes.results
/media/hayes/rsem_out/THXL1D.genes.results
/media/hayes/rsem_out/THXL1E.genes.results
/media/hayes/rsem_out/THXL1F.genes.results
/media/hayes/rsem_out/THXL7.genes.results
/media/hayes/rsem_out/THXL8.genes.results
/media/hayes/rsem_out/THXL9.genes.results
/media/hayes/rsem_out/THXL10.genes.results
/media/hayes/rsem_out/THXL11.genes.results
/media/hayes/rsem_out/THXL12.genes.results --out_prefix genes

# Annotate genes
genes = open('genes.counts.matrix')
ref = open('laevis.txt')
out = open('annotated.txt','w')

ref_dict = {}
```

```
for line in ref:
    entries = line.rstrip().split("\t")
    key = int(entries[0])
    symbol = entries[3]
    ref_dict[key] = symbol

header = True
for line in genes:
  if header:
    header = False
    out.write(line.rstrip() + "\tSymbol\n")
  else:
    key = -1
    try:
      key = int(line.split("\t")[0].split("|")[1])
    except ValueError:
      key = -1
    if key in ref_dict:
      out.write(line.rstrip() + "\t" + ref_dict[key] + "\n")
    else:
      out.write(line.rstrip() + "\tND\n")
```

Differential expression (DE) analysis

Statistical analysis was conducted using edgeR, a Bioconductor package for differential expression analysis of digital gene expression data (Robinson, McCarthy, & Smyth, 2010). `genes.counts.symbols` was converted to a `data.frame` object recognized by R. A `DGEList`, a list-based data object composed of a matrix (`counts`) containing the integer counts, a data frame (`samples`) containing information about the samples or libraries, and a data frame (`genes`) containing annotation for the genes or genomic features was created. We then performed trimmed mean of M-values (TMM) normalization, which adjusted for RNA composition by finding a set of scaling factors for the library sizes that minimize the log-fold changes between the samples for most genes. A normalization factor below one indicates that a small number of high count genes dominated the sequencing, resulting in lower counts for other genes. To adjust for this, the library size would be scaled down. Conversely, a factor above one would scale up the library size.

Next, we created a metadata table which describes our samples and their conditions. This information was used to setup a design matrix. edgeR uses the Cox-Reid profile-adjusted likelihood (CR) method to estimate dispersions. We plotted samples on a two-dimensional scatterplot so that distances on the plot approximate the expression differences between the samples. Additionally, we plotted the gene-wise biological coefficient of variation (BCV) against gene abundance (in log2 counts per million). Using the raw counts, dispersion, and design matrix, we fitted the negative binomial generalized linear model (GLM) for each gene and performed likelihood ratio tests with the several contrasts. We then generated a list of differentially expressed genes with false discovery rates (FDR) less than 0.06 for each comparison.

```
library(edgeR)
setwd("/Users/HayesLab/Projects/RNASeq/")

# Access count matrix and convert to R-readable file
rawdata <- read.table(file="genes.counts.symbols.txt",
header=TRUE, sep="\t", row.names=1)

# TMM normalization
dgeData <- calcNormFactors(DGEList(counts=rawdata[,2:13],
genes=rawdata[,1]))

# Create meta data table
meta <- read.table("meta.txt")

# Create appropriate groups, design, and contrasts
group <- factor(paste(meta$pop,meta$treat,sep="."))
cbind(meta,group=group)
design <- model.matrix(~0+group)
dispData <- estimateDisp(dgeData, design, robust=TRUE)
colnames(design) <- levels(group)
fit <- glmFit(dispData, design)
contrasts <- makeContrasts(
    XE.E2vsCON = XE.E2-XE.CON,
    SD.E2vsCON = SD.E2-SD.CON,
    SDvsXE.CON = SD.CON-XE.CON,
    SDvsXE.E2 = (SD.E2-SD.CON)-(XE.E2-XE.CON),
levels=design)

# Perform different contrasts
```

```
lrt_pop <- glmLRT(fit, contrast=contrasts[,"SDvsXE.CON"])
lrt_XEtreat <- glmLRT(fit, contrast=contrasts[,"XE.E2vsCON"])
lrt_SDtreat <- glmLRT(fit, contrast=contrasts[,"SD.E2vsCON"])
lrt_treat <- glmLRT(fit, contrast=contrasts[,"SDvsXE.E2"])
DE_pop <- topTags(lrt_pop, n=30000, adjust.method="BH", sort.by
= "logFC", p.value=0.06)
DE_XEtreat <- topTags(lrt_XEtreat, n=30000, adjust.method="BH",
sort.by = "logFC", p.value=0.06)
DE_SDtreat <- topTags(lrt_SDtreat, n=30000, adjust.method="BH",
sort.by = "logFC", p.value=0.06)
DE_treat <- topTags(lrt_treat, n=30000, adjust.method="BH",
sort.by = "logFC", p.value=0.06)

# Perform nested ANOVA
meta_nest <- read.table("meta.txt")
meta_nest$pop <- relevel(meta_nest$pop, ref="XE")
design_nest <- model.matrix(~pop * treat, data=meta_nest)
dispData_nest <- estimateDisp(dgeData, design_nest, robust=TRUE)
fit_nest <- glmFit(dispData_nest, design_nest)
lrt_poptreat_nest <- glmLRT(fit_nest, coef=4)
DE_poptreat_nest <- topTags(lrt_poptreat_nest, n=30000,
adjust.method="BH", sort.by = "logFC", p.value=0.06)

# Write data to .xlsx and .txt files
library(xlsx)

write.xlsx(dgeData$samples, "dgeData_samples.xlsx")

write.xlsx(DE_pop, "DE_pop.xlsx")
write.table(DE_pop, "DE_pop.txt", sep="\t")
write.xlsx(DE_XEtreat, "DE_XEtreat.xlsx")
write.table(DE_XEtreat, "DE_XEtreat.txt", sep="\t")
write.xlsx(DE_SDtreat, "DE_SDtreat.xlsx")
write.table(DE_SDtreat, "DE_SDtreat.txt", sep="\t")
write.xlsx(DE_treat, "DE_treat.xlsx")
write.table(DE_treat, "DE_treat.txt", sep="\t")

write.xlsx(DE_poptreat_nest, "DE_poptreat_nest.xlsx")
write.table(DE_poptreat_nest, "DE_poptreat_nest.txt", sep="\t")

# Print MDS plot and BCV plot
pdf('MDS_plot.pdf', width=8.5, height=11)
par(pin=c(6.5,6.5), omi=c(3,1,1,1), mar=c(4,4,2,0.5))
plotMDS(dgeData, labels = group)
dev.off()
```

```
pdf('BCV_plot.pdf', width=8.5, height=11)
par(pin=c(6.5,6.5), omi=c(3,1,1,1), mar=c(4,4,2,0.5))
plotBCV(dispData)
dev.off()
```

`DE_pop` examined any genes that responded differently to population between control animals; logFC values are in reference to XE. `DE_SDtreat` discovered genes that are differentially expressed between SD-E2 and SD-CON, while `DE_XEtreat` compared XE-E2 to XE-CON; logFC values are in reference to CON. `DE_treat` and `DE_poptreat_nest` took into account only E2-treated animals from both populations with treatment nested in population; both analyses yielded the same results; logFC values are in reference to XE-E2.

Gene ontology (GO) enrichment analysis

We used the Database for Annotation, Visualization, and Integrated Discovery (DAVID), a publicly available bioinformatics resource that extracts biological features associated with large gene lists (Huang, Sherman, & Lempicki, 2009).

RESULTS

Differential expression (DE) analysis

Table 1 displays the library size or sequencing depth for each sample and their corresponding normalization factors.

The multidimensional scaling plot of distances between samples (Figure 1) approximates the typical log2 fold changes between the samples. The common dispersion for the dataset is 0.3714709. The square root of the common dispersion gives the coefficient of biological variation (BCV), 0.61 (Figure 2).

When performing analysis, only differentially expressed genes with FDR <0.06 from each contrast were selected. *ahr.S*, *cyp2c8.2.L*, *cyp19a1.L*, *dmrt1.S*, and *hsd17b7.S* are genes recognized to have a role in sex differentiation and steroidogenesis. When comparing SD-CON and XE-CON animals, there were 152 genes that were unique, annotated, and

differentially expressed (FDR < 0.06); the expression of *ahr.S* and *hsd17b7.S* were 4.17 log2 fold (FDR = 0.0092) and 7.28 log2 fold (FDR = 2.32E-11) greater in SD males than in XE males, respectively (Table 2); the expression of *cyp2c8.2.L* was 3.55 log2 fold (FDR = 0.0067) greater in XE males than in SD males, respectively (Table 2). Six genes were unique, annotated, and differentially expressed (FDR < 0.06) between SD-E2 and SD-CON; *cyp19a1.L* expression was 9.83 log2 fold greater (FDR = 0.019), while *dmrt1.s* expression was 7.12 log2 fold lower (FDR = 0.059) in SD-E2 as compared to SD-CON (Table 3). In the XE population, there were 22 unique, annotated, differentially expressed genes (FDR < 0.06) between E2 and CON groups; *hsd17b7.S* expression was 8.25 log2 fold greater (FDR = 3.01E-12) in XE-E2 than in XE-CON (Table 4). When comparing E2-treated animals from both populations, there were 12 unique, annotated, and differentially expressed genes; *hsd17b7.S* expression was 8.50 log2 fold greater (FDR = 8.60E-11) in XE-E2 than in SD-E2 (Table 5).

Gene ontology (GO) enrichment analysis

After performing functional analysis for all contrasts, only one, SD-CON and XE-CON (`DE_pop`), had differentially expressed genes that showed clustering. Of the 16 clusters, one molecular function was significant: nucleotide binding (Table 6). The other non-significant clusters include: actin-binding, cytoskeleton regulation and binding, hydrolase activity, protease activity, cell cycle regulation, chromosomal regulation, GTP signaling, DNA binding, RNA biosynthesis and metabolism, protein localization, macromolecular complex assembly, protein kinase activity, ion transport, and signaling peptides.

DISCUSSION

We have characterized large disparities in E2 susceptibility among populations of *X. laevis* frogs; SD tadpoles showed higher female-to-male sex reversal rates than XE tadpoles and adult SD and XE males show different degrees of vitellogenin upregulation any given E2 treatment concentration. Here, we compared gonad gene expression between SD and XE animals during sex differentiation in response to E2 exposure. Because

the study animals were sacrificed prior to complete gonad differentiation, it was not possible to confirm the final sex ratio of each group. However, from previous sex ratio studies (Chapter Two), we hypothesized that compared to XE animals, a higher proportion of SD tadpoles would have been sex-reversed and developed into functioning females if they had been allowed to mature.

Of the genes found to be differentially expressed, three are known to be critical in steroid hormone biosynthesis: *cyp2c8.2.L* (EC: 1.14.14.1), *cyp19a1.L* (EC: 1.14.14.14), and *hsd17b7.S* (EC: 1.1.1.62). Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa, Sato, Kawashima, Furumichi, & Tanabe, 2016), we have highlighted these enzymes along the pathway (Figure 3). *cyp2c8.2.L* codes for a cytochrome P450 enzyme with monooxygenase activity, converting dehydroepiandrosterone (DHEA) into 16*α*-hydroxy-DHEA, a compound that activates ERβ activity (Miller et al., 2013). This enzyme also has dehydrogenase activity and is able to oxidize E2 into E1 (Cheng et al., 2001). SD-CON males had lower expression of *cyp2c8.2.L* than XE-CON males. As previously discussed, *cyp19a1.L* codes for aromatase, which converts androgens into estrogens. SD-E2 had increased expression of aromatase compared to SD-CON. *hsd17b7.S* encodes for the enzyme, hydroxysteroid (17-β) dehydrogenase (HSD17B7), which mediates conversion between E2 and estrone (E1) and between estriol (E3) and 16-α-hydroxyestrone (both reversible reactions) and of 4-androsten-11β-ol-3,17-dione into 11β-hydroxytestosterone (unidirectional). It is present in rat ovaries (Nokelainen, Peltoketo, Vihko, & Vihko, 1998), highly expressed in human ductal carcinoma and breast cancer cell lines, and is increased at the level of mRNA, protein expression, and promoter activity in MCF-7 cells in response to E2 exposure (Shehu et al., 2011). SD-CON males had higher expression of *hsd17b7.S* than XE-CON males, while XE-E2 had higher *hsd17b7.S* expression than XE-CON and SD-E2.

SD-CON males had higher expression of *ahr.S*, which codes for the aryl hydrocarbon receptor (AhR). The determination of activated-AhR action is complex and heavily ligand-dependent. AhR binds to several known endocrine disruptors such as polychlorinated dibenzodioxins

(dioxins), biphenyls (PCB), and polycyclic aromatic hydrocarbons (PAHs) (Poland & Knutson, 1982) and also has a critical role in female gonad development. Activation of AhR is very similar to that of nuclear receptors, in that upon ligand binding, there are conformation changes that lead to the dimerization and nuclear translocation of the receptor, resulting in changes in gene transcription (Swanson, 2002). The P450 enzymes such as *cyp1a2*, *cyp3a4*, *cyp1a1*, and *cyp1b1*, which are highly involved in the metabolism of E2 via hydroxylation, are the main targets of AhR (Tsuchiya, Nakajima, & Yokoi, 2005). Dioxin-activated AhR may "hijack" and activate ER in the absence of E2, upregulating the expression of E2-responsive genes (Brosens & Parker, 2003) such as *cyp19* in mice granulosa cells (Baba et al., 2005) and *in vitro* in three breast carcinoma cell lines and increased estrogen synthesis in the MCF-7 cell line (Saito et al., 2016). Conversely, in the presence of E2, dioxin activation of AhR impairs the expression of these same genes (Astroff, Eldridge, & Safe, 1991; Ohtake et al., 2003; Palumbo, Denison, Doroshov, & Tjeerdema, 2009) and increases the degradation of ER via proteasome activation (Wormke et al., 2003). In the absence of E2, dioxin-bound AhR recruits ERα to AhR-regulated genes, *cyp1a1* and *cyp1b1* (Matthews, Wihlen, Thomsen, & Gustafsson, 2005). Physiologically, AhR supports ovary development as AhR knockout (AhRKO) mice had significantly slower-growing follicles, lower serum and follicle-produced E2 levels, and decreased ER mRNA levels compared to wild-type mice (Barnett et al., 2007). AhR injection back into AhRKO follicles restored growth patterns comparable to wild-type levels and significantly increased expression of levels of AhR-targeted genes (Ziv-Gal, Gao, Karman, & Flaws, 2015). Futhermore, higher levels of follicle-stimulating hormone (FSH) was needed to induce maximal growth in AhR knockout mice compared to wild-type mice (Hernandez-Ochoa et al., 2013).

Lastly, SD-E2 males showed decreased expression of *dmrt1.S*, a transcription factor involved in testis formation in many vertebrates (Yoshimoto et al., 2006), compared to SD-CON. It serves as an upregulator of several important genes involved in male sex determination and gonad differentiation. Suppression of expression of DMRT1 further pushes SD

males towards feminization and away from male differentiation. These data confirm estrogen's inhibitory action on *dmrt1.s* transcription and enhancement of its own production by upregulating aromatase in SD males. Furthermore, our findings suggest that differences in susceptibility to feminization occurs upstream of differentiation and stems at the level of early sex determination.

Based on the expression profiles above, we propose the following mechanism underlying susceptibility to feminization, which involves both the suppression of masculinizing genes and the upregulation in feminizing genes (Figure 4). In the absence of E2, SD males were "pre-disposed" to feminization due to higher expression of ovary-supporting genes, *ahr.S* (important in follicle development) and *hsd17b7.S* (E1 to E2 conversion), and lower expression of *cyp2c8.2.L* (E2 to E1 conversion). After E2 exposure, SD males progressed further towards female development by downregulating a masculinizing gene, *dmrt1.s*, and increasing a feminizing gene, *cyp19a1.L*. Decreased susceptibility of XE animals was associated with increased *hsd17b7.S* expression, which in this population, may have been metabolizing E2 to E1 in the presence on high exogenous E2. Future quantitative real-time polymerase chain reaction (qPCR) experiments must be conducted in E2-treated gonads of animals from both populations to confirm observed changes in expression of *ahr.S*, *cyp2c8.2.L, cyp19a1.L, hsd17b7.S*, and *dmrt1.S*.

ACKNOWLEDGEMENTS

Special thanks to Christopher J. Sifuentes, PhD of the Laboratory of Developmental Neuroendocrinology at the University of Michigan, Ann Arbor for his advice and expertise on RNA-Seq data analysis and to John C. Wright of the Berkeley Wireless Research Center at the University of California, Berkeley for his support writing scripts for the pre-processing of sequencing data. This work used the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley, supported by NIH S10 Instrumentation Grants S10RR029668 and S10RR027303.

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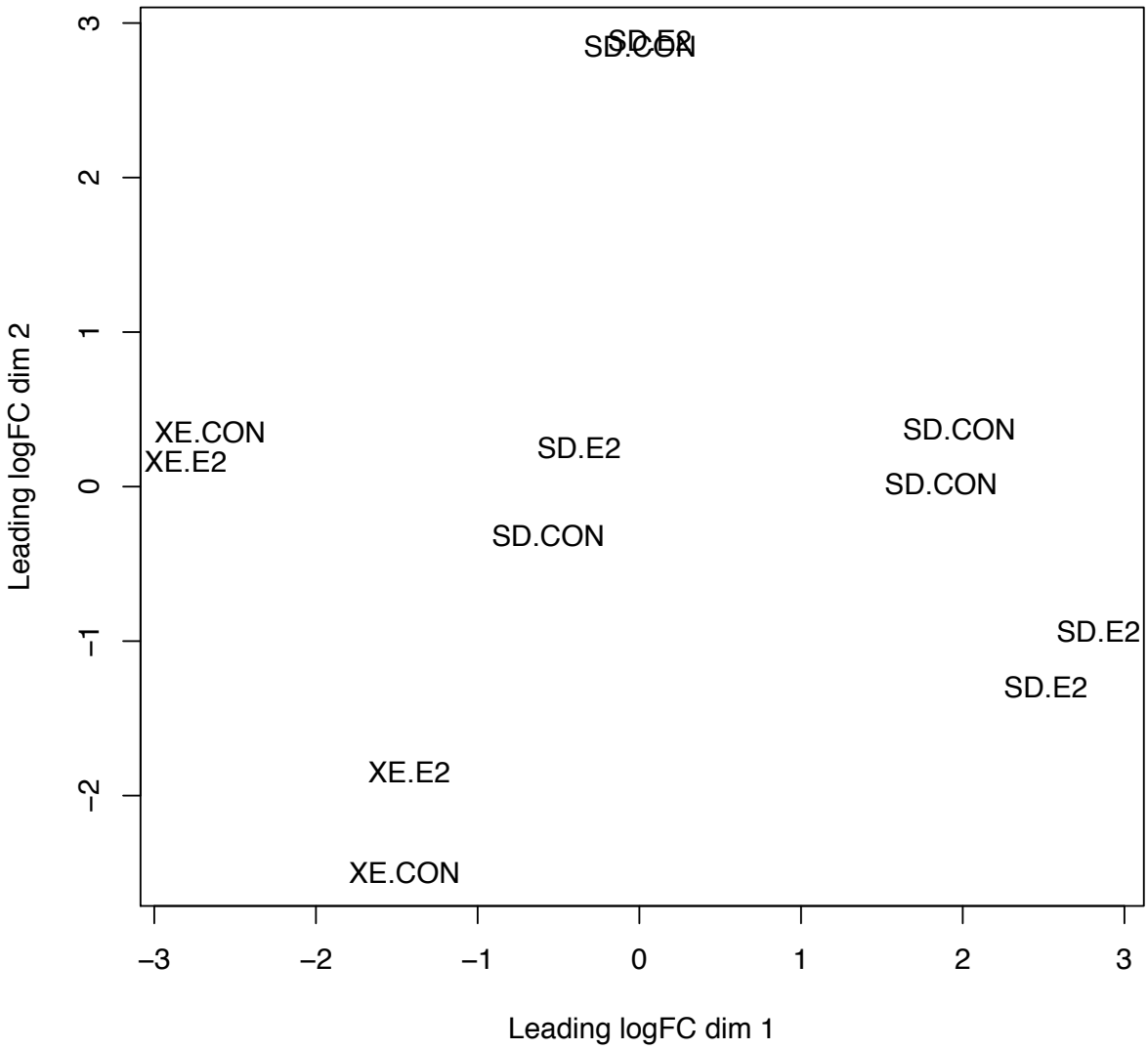


Figure 1 Multidimensional scaling (MSD) plot. The distances between samples approximate the typical log2 fold changes between the samples.

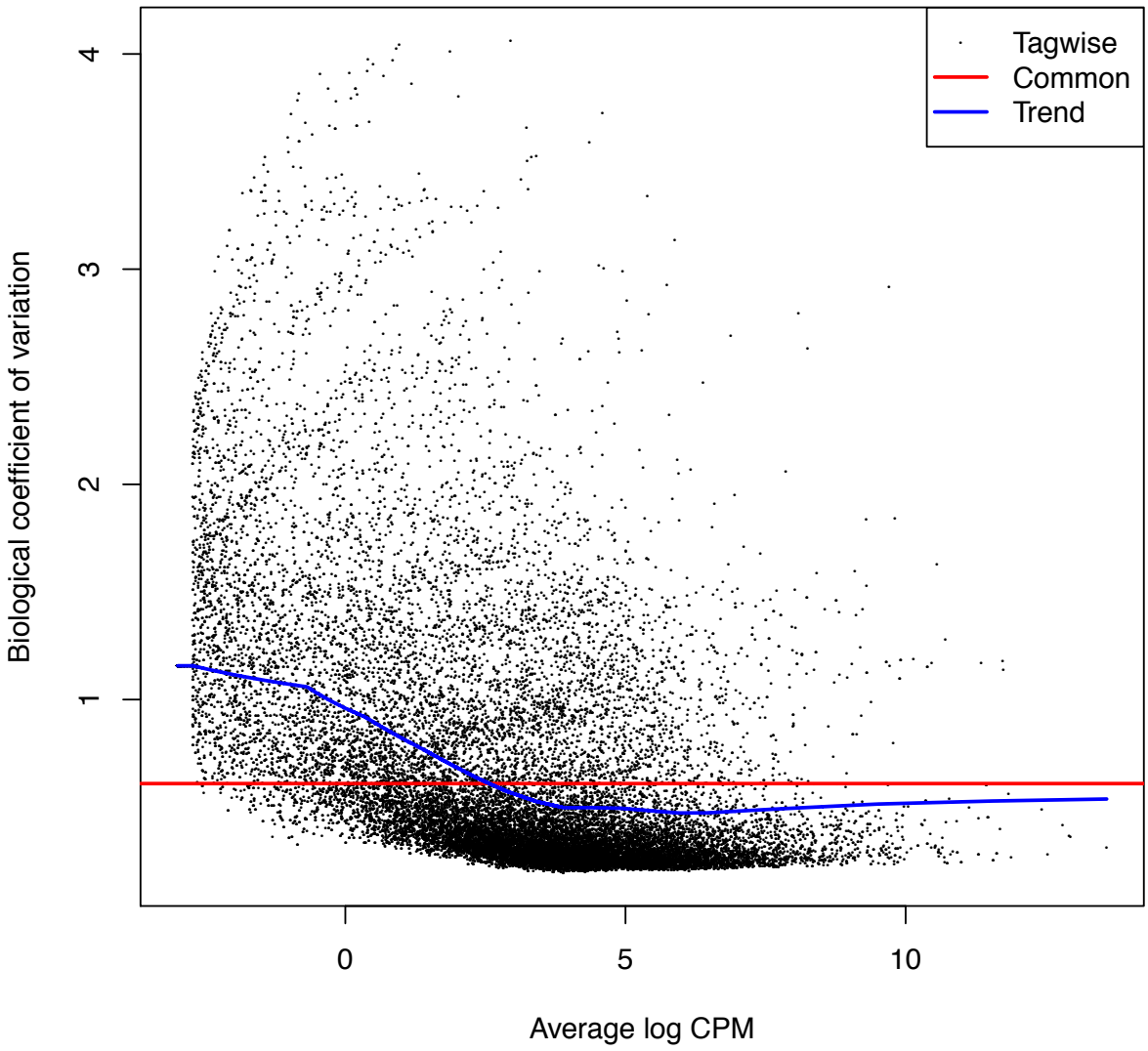


Figure 2 Gene-wise biological coefficient of variation (BCV) plot. The BCV for every gene is plotted against gene abundance (in log2 counts per million).

Sample name	lib.size	norm.factors
THXL1A	14515941.02	0.916300117
THXL1B	16461901.41	0.942625385
THXL1C	13883300.35	0.933608001
THXL1D	17975436.29	1.06526345
THXL1E	13680604.83	0.951381834
THXL1F	17794470.91	0.927368168
THXL7	16451181.15	1.086849468
THXL8	15319063.25	0.936974764
THXL9	16432719	1.099552386
THXL10	16303942.33	1.070423104
THXL11	13973200.67	1.036242911
THXL12	17243237.05	1.06234171

Table 1 Sizes and normalization factors of libraries. A normalization factor below one indicates that a small number of high-count genes dominated the sequencing, resulting in lower counts for other genes. To adjust for this, the library size will be scaled down. Conversely, a factor above one will scale up the library size.

Gene symbol	logFC	logCPM	LR	PValue	FDR
vtn.L	-14.12663525	4.589432343	12.58295427	0.000389281	0.047257465
epx.L	14.00319947	9.779285851	51.25327188	8.11853E-13	6.89895E-10
nptn.L	13.18139601	5.74244444	156.389934	6.95759E-36	5.32117E-32
srsf1.L	-12.76849433	4.299666389	148.6709582	3.38427E-34	2.07063E-30
prkaca.L	-12.06520819	3.130912735	190.0324594	3.12785E-43	4.78436E-39
ND	-11.97183013	4.016515088	127.3685897	1.54286E-29	4.29083E-26
ctnna1.S	-11.60870534	2.060818644	118.6949037	1.22143E-27	3.11383E-24
mef2d.S	11.567344	4.120364417	24.77310219	6.44912E-07	0.000205382
ND	-11.53184016	2.898987763	12.18012208	0.000483015	0.055089721
gata6.L	11.42304172	3.835434028	93.89479391	3.32748E-22	7.83033E-19
prep.L	11.32454994	3.948736481	90.77759916	1.60763E-21	3.5129E-18
cmtm6.S	-11.1845975	2.418411034	17.40448309	3.02113E-05	0.005886769
aplnr.L	-11.17357774	2.540374772	61.3241668	4.84102E-15	5.48505E-12
esf1.L	10.77525106	3.138409104	12.18084771	0.000482827	0.055089721
slc5a8.L	-10.75626317	1.748239534	16.43352658	5.03861E-05	0.008808072
ND	10.69492937	6.948796867	144.1082275	3.36456E-33	1.14365E-29
ND	10.69492937	6.948796867	144.1082275	3.36456E-33	1.14365E-29
ND	10.69492937	6.948796867	144.1082275	3.36456E-33	1.14365E-29
ND	10.69492937	6.948796867	144.1082275	3.36456E-33	1.14365E-29
hla-a.L	-10.58759717	1.813242872	21.34952349	3.82716E-06	0.001090869
ND	10.57279468	2.803991475	33.37414942	7.60278E-09	3.63413E-06
ND	-10.35800029	1.483505941	86.93138578	1.12352E-20	2.14817E-17
gtf2h4.S	10.13044774	3.167980088	31.63973986	1.85593E-08	8.73484E-06
skap2.L	10.08927788	2.575990896	41.11830401	1.43288E-10	9.13223E-08
pak3.L	-10.07460309	1.090729021	81.96291023	1.38665E-19	2.35668E-16
tom1.S	10.03438065	2.903265259	12.0378457	0.000521312	0.057992627
dnase1.L	-9.941039719	1.171487466	12.45894677	0.000415994	0.049711336
dnase1.L	-9.941039719	1.171487466	12.45894677	0.000415994	0.049711336
ND	9.905655099	2.59439483	12.37965255	0.000434038	0.050487026
yeats4.S	-9.819989965	1.787847064	18.4112581	1.78003E-05	0.003889624
tmem126a.L	9.772150118	2.499259065	30.68055521	3.04198E-08	1.40438E-05
unnamed.L	9.683963347	2.902296045	16.64837651	4.49888E-05	0.008095866
ND	9.576004301	2.033166736	27.89244483	1.2825E-07	4.78465E-05
prss1.L	9.511153582	2.262981699	15.26101643	9.36291E-05	0.015651912
ND	-9.501406012	3.532383066	156.6284126	6.17088E-36	5.32117E-32
ND	9.46133666	2.294513489	17.99066099	2.21991E-05	0.00465148
ND	-9.398493056	0.799619152	70.17495594	5.42711E-17	6.91776E-14

Gene symbol	logFC	logCPM	LR	PValue	FDR
ghr.L	9.391158036	1.976435352	23.99927721	9.63719E-07	0.000291902
ND	9.382812369	1.808584219	59.86007619	1.01847E-14	1.00506E-11
ND	9.382812369	1.808584219	59.86007619	1.01847E-14	1.00506E-11
ND	9.320549033	1.127487298	13.81163239	0.000202081	0.027997875
ND	9.320549033	1.127487298	13.81163239	0.000202081	0.027997875
ND	-9.318377033	1.335323193	28.87419385	7.72351E-08	3.15037E-05
tmcc1.L	9.317420591	2.122932185	13.39842345	0.000251836	0.032370434
hsp90aa1.1.L	-9.241840903	0.870861428	14.75322974	0.000122537	0.019729819
apoa5.L	-9.170654611	-0.248052405	13.73107029	0.000210936	0.02824334
galm.L	9.020095241	1.89305116	14.60117623	0.000132832	0.020946312
ND	-8.967990381	7.656039666	306.4773671	1.27826E-68	3.91046E-64
rtn4.L	-8.959480474	0.028042914	60.31065818	8.10076E-15	8.62452E-12
lysmd2.L	8.857120494	1.202221825	13.57789701	0.000228864	0.029875224
nr1h5.S	-8.733208716	0.441972836	13.29367668	0.000266303	0.033803873
rusc1.S	-8.701736698	-0.049156746	23.09638105	1.5408E-06	0.00046212
ralb.L	8.681464529	5.116893078	79.69996775	4.35808E-19	6.66612E-16
snrnp40.S	8.640351951	1.598418485	38.3520664	5.90655E-10	3.543E-07
fam96a.L	8.615430965	1.929950801	38.17971237	6.45198E-10	3.76232E-07
pdpk1.L	8.517105715	1.936688584	17.23428476	3.30419E-05	0.006317618
incenp.S	-8.402319343	2.827282101	80.40805855	3.04551E-19	4.90359E-16
gadd45g.L	-8.395191364	1.878184759	12.2520324	0.000464752	0.053651636
tgm2.L	-8.300734252	0.194199835	42.44347806	7.27546E-11	5.17607E-08
loc495492.L	-8.297686485	1.393317658	26.76900498	2.29286E-07	8.06242E-05
fen1.L	-8.251180452	-0.23471683	29.28933838	6.23377E-08	2.57708E-05
fen1.L	-8.251180452	-0.23471683	29.28933838	6.23377E-08	2.57708E-05
ahcy.L	-8.033839029	2.490669105	14.52776713	0.000138109	0.021338513
ahcy.L	-8.033839029	2.490669105	14.52776713	0.000138109	0.021338513
parp1.L	-8.010269065	3.297041607	24.34553834	8.0513E-07	0.000246305
ND	-8.005517503	-0.632905815	38.42786087	5.68155E-10	3.4762E-07
ND	7.992119458	0.711533076	12.1747339	0.000484412	0.055089721
tmsb4x.S	-7.98616723	-1.070121966	14.42623155	0.000145758	0.022295112
tmsb4x.S	-7.98616723	-1.070121966	14.42623155	0.000145758	0.022295112
ND	-7.92960264	0.150920534	41.25751709	1.33438E-10	8.68537E-08
ND	-7.92960264	0.150920534	41.25751709	1.33438E-10	8.68537E-08
c1orf109.L	7.918671007	0.146024867	25.44504554	4.55165E-07	0.000151352
ND	7.900646225	3.633217208	16.60250075	4.60902E-05	0.008150241

Gene symbol	logFC	logCPM	LR	PValue	FDR
pik3c3.L	7.857401174	4.395380096	13.66470078	0.000218524	0.028939735
znrf1.S	7.852390148	1.878632637	72.5803313	1.60369E-17	2.13305E-14
vangl2.L	-7.754832056	-1.296836304	28.4144583	9.79312E-08	3.74489E-05
enc1.2.L	-7.672412457	2.902723905	49.79855504	1.70369E-12	1.40863E-09
rad17.L	7.642032437	2.801721165	19.62589377	9.41841E-06	0.002361705
smc4.L	7.527725586	3.569727003	128.0369169	1.10174E-29	3.37046E-26
hla-a.L	-7.408534667	-0.454480665	36.69707453	1.37986E-09	7.40575E-07
c1qtnf9b.L	-7.406103392	0.963259073	16.80497541	4.14246E-05	0.007588382
tpst2.L	7.371166955	4.01028465	27.57108461	1.51426E-07	0.000054499
cfap70.L	-7.301887154	-0.860713383	14.26845773	0.000158499	0.023768601
ND	7.281112025	4.110252271	41.95047384	9.36147E-11	6.36414E-08
hsd17b7.S	7.278224352	4.78882866	58.15352951	2.42439E-14	2.31772E-11
tctex1d1.S	-7.117769885	-0.430174829	13.2809467	0.000268117	0.033893543
ND	-7.070289778	0.000411099	14.15600968	0.000168259	0.024987244
bet1l.S	6.968202665	2.893504438	74.26082247	6.84485E-18	9.59413E-15
nudt15.L	-6.873026607	-0.699705962	13.90315468	0.000192475	0.02771709
spry1.S	-6.865045696	-0.239692385	15.9094977	6.64445E-05	0.011419487
rprd1a.S	6.848231396	2.203506057	19.49595951	1.00813E-05	0.00246725
rprd1a.S	6.848231396	2.203506057	19.49595951	1.00813E-05	0.00246725
tp53bp2.L	-6.811500528	2.546363321	14.56156551	0.000135653	0.021281586
ND	-6.757852067	-1.213453141	28.4513983	9.60803E-08	3.72062E-05
ND	-6.757852067	-1.213453141	28.4513983	9.60803E-08	3.72062E-05
icmt.S	6.736940763	-0.971009562	14.7697364	0.000121469	0.019729819
icmt.S	6.736940763	-0.971009562	14.7697364	0.000121469	0.019729819
oaz2.S	6.69222683	-0.51216393	20.70939969	5.3453E-06	0.001409684
oaz2.S	6.69222683	-0.51216393	20.70939969	5.3453E-06	0.001409684
txn.L	6.589664101	6.705237006	12.15706163	0.000489024	0.055408183
ND	-6.554753524	2.175156968	15.7673942	7.16263E-05	0.012241301
ND	-6.524683925	-0.844516308	21.78599199	3.04818E-06	0.000879715
ND	-6.282450989	-2.131012786	19.07702029	1.25548E-05	0.002909659
ND	-6.26771451	-1.933795636	14.85678568	0.00011599	0.019077242
rtn4.S	-6.248109026	3.798294736	28.49629182	9.38781E-08	3.72062E-05
ND	-6.217933414	-1.281537627	20.78218002	5.14596E-06	0.001380922
ND	-6.217933414	-1.281537627	20.78218002	5.14596E-06	0.001380922
ND	-6.205130346	0.242564762	34.44543472	4.3837E-09	2.12867E-06
ND	6.032709582	8.801932558	87.64603475	7.82813E-21	1.59652E-17
ND	-6.006417062	-2.233034338	18.21604904	0.000019721	0.004248626

Gene symbol	logFC	logCPM	LR	PValue	FDR
ND	-5.978729271	0.828719369	37.27655794	1.02509E-09	5.59994E-07
gtf3a.L	5.916158272	2.317720847	22.06087365	2.64139E-06	0.000769576
ND	-5.771230133	-0.286960016	24.48785093	7.47799E-07	0.000231077
ND	-5.69767416	-1.695787284	17.5659099	2.77519E-05	0.00554893
ND	-5.69767416	-1.695787284	17.5659099	2.77519E-05	0.00554893
hint1.S	5.645371785	4.111712812	24.72141791	6.62439E-07	0.000206789
ND	5.639570336	-1.157584121	12.85412528	0.000336736	0.041538024
ND	5.639570336	-1.157584121	12.85412528	0.000336736	0.041538024
ND	-5.602776342	-0.339326805	27.62642557	1.47155E-07	5.35923E-05
elavl2.S	5.310571751	0.280717083	14.53535037	0.000137554	0.021338513
s100a11.L	5.307598917	5.449359813	27.63309578	1.46648E-07	5.35923E-05
pop7.L	-5.299005019	0.820274283	19.04314977	1.27796E-05	0.002917557
pop7.L	-5.299005019	0.820274283	19.04314977	1.27796E-05	0.002917557
scg2.L	5.273632488	1.687436497	14.76036528	0.000122075	0.019729819
ND	5.245184614	5.736782308	19.09944559	1.24081E-05	0.002897621
mhc1a.L	-5.160034096	-0.729987123	18.49831812	1.70054E-05	0.003769783
mhc1a.L	-5.160034096	-0.729987123	18.49831812	1.70054E-05	0.003769783
ND	-5.100533264	-0.078921437	15.66760609	7.55064E-05	0.012832728
ND	-4.997523404	-0.036434705	36.40159255	1.60572E-09	8.32579E-07
ND	-4.997523404	-0.036434705	36.40159255	1.60572E-09	8.32579E-07
hsp90b1.L	-4.985438491	2.251791775	13.63783556	0.000221673	0.029104786
hsp90b1.L	-4.985438491	2.251791775	13.63783556	0.000221673	0.029104786
hla-a.L	-4.862284156	2.60385611	45.58329498	1.46284E-11	1.0655E-08
bckdhb.S	4.825891646	5.5270623	16.4635411	4.95947E-05	0.008719542
fech.L	-4.588108663	1.276138043	19.81801599	8.51761E-06	0.002153476
chrng.L	-4.548276059	-0.192226767	30.42979051	3.46171E-08	1.53479E-05
ND	4.531010148	2.370064052	36.24870319	1.73676E-09	8.85514E-07
ap3b1.L	4.528615254	5.201840637	19.53885606	9.85741E-06	0.00245169
efnb1.L	-4.524493376	-0.927032606	12.40032521	0.000429259	0.050487026
efnb1.L	-4.524493376	-0.927032606	12.40032521	0.000429259	0.050487026
ndufc2.S	4.462281651	2.620311957	36.17196604	1.80651E-09	8.91365E-07
ndufc2.S	4.462281651	2.620311957	36.17196604	1.80651E-09	8.91365E-07
crym.L	4.458228518	3.20466317	14.37217167	0.000150003	0.022717265
ND	-4.449343488	4.07648862	15.43504133	8.53901E-05	0.014353051
cebpa.L	-4.432352739	4.330624388	60.29253211	8.1757E-15	8.62452E-12
gng7.L	-4.392745989	1.453166343	17.78823083	2.46906E-05	0.005107726
gng7.L	-4.386811523	1.410987967	17.78669683	2.47105E-05	0.005107726

Gene symbol	logFC	logCPM	LR	PValue	FDR
brd2.S	-4.384276249	3.585328831	74.24511294	6.89954E-18	9.59413E-15
ccnh.L	4.300538871	1.600421256	17.24846308	3.27963E-05	0.006310086
ND	-4.190164577	0.767732068	46.04829001	1.15374E-11	8.82379E-09
ND	4.189107326	3.844317153	12.38290116	0.000433283	0.050487026
casp2.L	4.179600413	4.646003775	14.02600448	0.0001803	0.026537345
ahr.S	4.169736236	3.677701419	16.33868096	5.29716E-05	0.009207427
ND	-4.039338777	1.105260663	16.82698729	4.09468E-05	0.007588382
sparc.S	4.025082455	7.312438812	19.40113086	1.05944E-05	0.00251244
sparc.S	4.025082455	7.312438812	19.40113086	1.05944E-05	0.00251244
adrm1.S	3.938609979	0.722684531	13.81514466	0.000201704	0.027997875
stau1.L	3.913319886	5.85925519	82.540912	1.03505E-19	1.86261E-16
hla-a.L	-3.857487717	2.555815971	53.91510583	2.09342E-13	1.94066E-10
wbp1.S	3.812522173	1.767849728	20.81668839	5.05406E-06	0.001380482
wbp1.S	3.812522173	1.767849728	20.81668839	5.05406E-06	0.001380482
ssh3.L	3.792767245	1.817162348	19.45206473	1.03156E-05	0.002484848
ssh3.L	3.792767245	1.817162348	19.45206473	1.03156E-05	0.002484848
ND	3.75597552	2.845409964	45.91816057	1.23298E-11	9.19981E-09
chrng.L	-3.67151139	-0.158716508	17.51842724	2.84537E-05	0.0056523
nme2.S	3.611752259	6.125901552	30.44143209	3.441E-08	1.53479E-05
nme2.S	3.602194229	6.125693063	30.39857855	3.51786E-08	0.000015374
cyp2c8.2.L	-3.548624945	3.347919123	17.10983443	3.52792E-05	0.006662098
cyp2c8.2.L	-3.548624945	3.347919123	17.10983443	3.52792E-05	0.006662098
ND	-3.546808771	1.000892103	12.75456383	0.000355142	0.043457962
atp1a1.L	-3.42860021	8.551654812	38.56691169	5.29083E-10	3.30321E-07
cfl1.S	-3.413829916	3.58019474	18.26979472	1.91723E-05	0.004159714
pdha1.S	-3.358989695	3.250216951	28.78445191	8.0898E-08	3.25636E-05
rhof.S	-3.310300664	0.896436485	24.7543424	6.51219E-07	0.000205382
ND	3.285804089	1.95506056	19.31731852	1.10698E-05	0.002604973
ube2l3.S	3.206062579	5.777296709	29.29313799	6.22156E-08	2.57708E-05
tubg1.L	3.189424842	1.820397907	25.39074862	4.68158E-07	0.00015236
tubg1.L	3.189424842	1.820397907	25.39074862	4.68158E-07	0.00015236
uqcrc2.L	-3.122487409	3.300313338	38.08753156	6.7641E-10	3.76232E-07
uqcrc2.L	-3.122487409	3.300313338	38.08753156	6.7641E-10	3.76232E-07
hist1h2ad.L	3.119202868	2.444171955	29.38996243	5.91834E-08	2.55005E-05
psmc5.L	3.042355477	6.019092655	13.38677795	0.000253404	0.032435754
gtf2f1.L	-3.035471698	0.602752586	13.0925386	0.000296474	0.03732404
atp1b3.L	-3.016518864	3.028691646	64.61456244	9.10797E-16	1.07166E-12

Gene symbol	logFC	logCPM	LR	PValue	FDR
atp1b3.L	-3.016518864	3.028691646	64.61456244	9.10797E-16	1.07166E-12
ND	2.932718058	6.823342998	26.52085739	2.60708E-07	8.96133E-05
vim.S	2.929389992	6.416453607	38.13154168	6.61324E-10	3.76232E-07
pabpc1.S	2.907013542	5.614729356	22.79469574	1.80263E-06	0.000535397
mcm6.2.L	2.868444737	5.371701772	47.11995881	6.67723E-12	5.23769E-09
mcm6.2.L	2.868444737	5.371701772	47.11995881	6.67723E-12	5.23769E-09
cirbp.S	2.836276884	9.227167025	13.84654016	0.000198362	0.027997875
ND	2.817884956	1.771900751	13.77911947	0.000205609	0.027997875
mtx1.S	2.752538902	3.175369545	26.71884145	2.35316E-07	8.18043E-05
hmgb2.S	-2.722208194	6.314201399	30.65912836	3.07576E-08	1.40438E-05
ND	-2.711621529	3.440499841	27.0544543	1.97804E-07	0.000070363
rara.L	-2.683188551	2.557772393	18.01296517	2.19406E-05	0.004629004
camk2g.S	2.66712125	4.155160058	26.44125917	2.71675E-07	9.23454E-05
atp1a1.L	-2.55322947	7.532379922	20.41874413	6.22174E-06	0.001613013
atp1a1.L	-2.55322947	7.532379922	20.41874413	6.22174E-06	0.001613013
ND	2.537268191	5.794842982	13.82219069	0.000200949	0.027997875
rnd1.L	-2.45414354	1.505938501	13.78234428	0.000205256	0.027997875
tcf7l1.L	2.453863808	0.775740783	13.51605122	0.000236532	0.030531539
tcf7l1.L	2.453863808	0.775740783	13.51605122	0.000236532	0.030531539
anp32b.L	-2.452235032	2.423771425	13.96887218	0.000185862	0.027075735
tmem51.S	2.44079869	1.532677131	13.72677534	0.000211419	0.02824334
tmem51.S	2.44079869	1.532677131	13.72677534	0.000211419	0.02824334
e2f8.L	2.405486574	2.501216526	14.61414257	0.000131921	0.020910473
e2f8.L	2.405486574	2.501216526	14.61414257	0.000131921	0.020910473
acaa1.L	-2.401644038	5.582450234	20.18324481	7.03666E-06	0.001808953
acaa1.L	-2.388872116	5.608785652	18.44409566	1.74962E-05	0.003850676
hla-a.L	-2.339860848	2.102519162	12.13270955	0.000495451	0.055929267
ND	-2.323300257	1.268742219	15.01425525	0.000106702	0.017740387
odc1.L	2.313111381	7.982318295	42.27985356	7.91032E-11	5.49983E-08
ND	-2.26156096	2.340073746	20.98733056	4.62331E-06	0.001285783
prickle1.L	-2.253013103	1.554151213	13.77931825	0.000205587	0.027997875
prickle1.L	-2.253013103	1.554151213	13.77931825	0.000205587	0.027997875
rab18.S	-2.241581985	5.171843378	25.21290433	5.13375E-07	0.000165318
xnf7.L	-2.180505918	4.349188415	12.51918249	0.000402795	0.048513022
nme2.S	-2.173224957	5.44278065	17.56953464	2.76991E-05	0.00554893
ND	-2.167115787	2.719020297	28.146381	1.12478E-07	4.24805E-05
ND	2.161068353	3.609923437	26.16865036	3.12861E-07	0.000105176

Gene symbol	logFC	logCPM	LR	PValue	FDR
ND	2.136448042	2.435671993	13.88602975	0.000194237	0.02771709
ND	2.136448042	2.435671993	13.88602975	0.000194237	0.02771709
ctsd.S	-2.13328564	4.825771904	51.77414968	6.22662E-13	5.44242E-10
ctsd.S	-2.13328564	4.825771904	51.77414968	6.22662E-13	5.44242E-10
ND	2.104516108	4.797586875	13.68046425	0.000216697	0.028822589
cers3.S	-2.097627973	4.603838999	17.64385928	2.66374E-05	0.005432607
ND	2.06837712	7.26299713	12.07573408	0.000510823	0.057033253
acta2.S	2.06837712	7.26299713	12.07573408	0.000510823	0.057033253
rbm38.L	2.056875899	3.975038315	17.31075031	3.17386E-05	0.00614524
ND	2.051783522	9.612261391	21.19845892	4.14097E-06	0.001162208
entpd3.L	-2.024600932	1.053149389	14.6944471	0.000126418	0.020248106
prickle1.L	2.021848542	2.802950308	13.57274187	0.000229494	0.029875224
calcoco1.S	-2.012935767	2.458461856	20.06723566	7.47666E-06	0.001906051
ND	-1.978384293	3.492576904	13.94904794	0.000187833	0.027233079
snap23.L	-1.84876286	2.463761539	16.6032626	4.60717E-05	0.008150241
snap23.L	-1.84876286	2.463761539	16.6032626	4.60717E-05	0.008150241
ND	-1.832995865	2.319743547	13.34857995	0.000258618	0.032965223
acly.S	1.797096522	4.613322911	15.59293418	7.85476E-05	0.01327584
pold3.L	-1.743483252	2.375352842	12.39181341	0.00043122	0.050487026
pold3.L	-1.743483252	2.375352842	12.39181341	0.00043122	0.050487026
ND	1.649878046	5.941071006	12.2762186	0.000458767	0.053161363
ptpn11.S	1.645435353	3.526289217	18.0273239	2.17757E-05	0.004626125
ptpn11.S	1.645435353	3.526289217	18.0273239	2.17757E-05	0.004626125
pccb.L	-1.610348211	6.041733746	21.3375522	3.85113E-06	0.001090869
yif1b.S	1.579046009	5.12590645	22.27620291	2.36115E-06	0.00069454
txnl4a.S	1.571288344	4.206116166	16.76225563	4.23677E-05	0.007669309
txnl4a.S	1.571288344	4.206116166	16.76225563	4.23677E-05	0.007669309
wsb1.L	-1.566932858	5.273254155	17.76963794	2.49331E-05	0.00511914
cd74.S	-1.556478424	3.991800924	16.94308611	3.85173E-05	0.007228973
pmf1.L	1.549373134	4.382681399	12.10207658	0.000503657	0.056646599
rac3.S	1.538658157	5.663905306	12.83855927	0.000339549	0.041716794
ubxn1.S	1.5376544	5.562409872	12.23810707	0.000468233	0.053850324
fam64a.L	1.537116666	4.816004997	18.68310167	1.54344E-05	0.003497556
tp53.L	1.534039371	5.238064112	16.28062865	5.46195E-05	0.009440222
metap2.S	1.524341073	3.264475763	12.54984053	0.00039624	0.047912164
cpvl.L	-1.502237651	4.275812568	13.7679422	0.000206836	0.027997875
cpvl.L	-1.502237651	4.275812568	13.7679422	0.000206836	0.027997875

Gene symbol	logFC	logCPM	LR	PValue	FDR
rnasek.L	-1.499290026	3.258367732	12.93332264	0.000322784	0.040140725
rnasek.L	-1.499290026	3.258367732	12.93332264	0.000322784	0.040140725
ND	1.451542984	6.225443828	17.44864997	2.95174E-05	0.005788435
ND	1.451542984	6.225443828	17.44864997	2.95174E-05	0.005788435
hmgn1.L	1.445273224	6.793463769	12.69520717	0.000366594	0.04468065
ND	1.37906659	3.528388151	14.22155951	0.000162498	0.024249467
nfu1.S	1.37519331	3.582806305	12.39195093	0.000431189	0.050487026
prkar1a.S	1.345680484	7.691125311	14.41120066	0.000146926	0.022361962
cgnl1.L	-1.323781778	5.488467152	14.99579687	0.000107751	0.017817922
vim.L	-1.311686244	5.748843935	16.80957783	4.13242E-05	0.007588382
brd2.S	1.295015283	5.221350447	13.04608092	0.00030392	0.038104556
mcm6.2.L	-1.2835633	6.101620495	18.52768686	1.67454E-05	0.003766731
ND	-1.263681383	7.362852842	14.34004296	0.000152585	0.022994414
mrpl10.S	1.257367748	4.431256356	13.88063555	0.000194795	0.02771709
ap1g1.L	-1.25341861	6.802781789	16.86067686	4.02264E-05	0.007503695
prkar1a.S	-1.030074439	7.175531904	14.01561058	0.000181299	0.026537345
prkar1a.S	-1.030074439	7.175531904	14.01561058	0.000181299	0.026537345

Table 2 Differentially expressed genes: `DE_pop` contrast. DE analysis between SD-CON and XE-CON males (FDR < 0.06).

Gene Symbol	logFC	logCPM	LR	PValue	FDR
cyp19a1.L	9.830690268	1.498816392	21.06852051	4.43149E-06	0.019366866
yeats4.S	9.620373119	1.787847064	18.87818066	1.39337E-05	0.053282593
ND	-9.320549033	1.127487298	26.68969033	2.38893E-07	0.003654104
ND	-9.320549033	1.127487298	26.68969033	2.38893E-07	0.003654104
ND	9.069072343	0.611766335	18.43871799	1.75457E-05	0.058986322
ND	7.412854766	1.335323193	21.114722	4.32592E-06	0.019366866
dmrt1.S	-7.121871386	1.604331897	18.25896649	1.92816E-05	0.058986322
ctu2.S	-6.967760184	1.153143443	22.15158931	2.51947E-06	0.015415144
polr2k.L	5.456261309	1.998850811	24.21812811	8.60188E-07	0.006578718
lyar.S	-1.977900522	4.033862372	24.27474422	8.35271E-07	0.006578718

Table 3 Differentially expressed genes: `DE_SDtreat` contrast. DE analysis between SD-E2 and SD-CON males (FDR < 0.06).

Gene symbol	logFC	logCPM	LR	PValue	FDR
ctnna1.S	-11.60870534	2.060818644	63.74956494	1.41285E-15	1.08055E-11
cutc.L	-10.93345404	3.68460083	16.33335357	5.31207E-05	0.051195365
gtf2h4.S	10.89201439	3.167980088	34.41921444	4.44315E-09	1.51028E-05
unnamed.L	10.73132025	2.902296045	18.71065878	1.5213E-05	0.01939147
ND	10.57435857	2.294513489	20.56954813	5.75036E-06	0.009258681
ND	-10.42072658	3.776122798	18.59051873	1.62024E-05	0.0198266
prss1.L	10.20018059	2.262981699	16.31804255	5.35516E-05	0.051195365
ND	-10.05790468	3.141446828	17.86807569	2.3676E-05	0.025867682
ND	-9.916486402	2.226816916	21.34410843	3.83798E-06	0.007827441
tcap.S	9.523682951	0.212507998	18.34828859	1.83984E-05	0.021647861
ctu2.S	-9.502875572	2.718735285	17.75946383	2.50667E-05	0.026442822
ND	-8.924783812	4.151489952	16.43891573	5.02431E-05	0.051195365
pef1.L	-8.860030313	3.868445671	19.94854001	7.95547E-06	0.01106244
ND	8.537949327	4.110252271	50.91688418	9.63611E-13	4.91313E-09
eed.L	-8.345103211	2.976903798	20.03616214	7.59914E-06	0.01106244
eed.L	-8.345102921	2.976912737	20.03692487	7.59611E-06	0.01106244
znrf1.S	8.324069112	1.878632637	78.51180434	7.95216E-19	2.43273E-14
fam96a.L	8.278440672	1.929950801	32.3585694	1.28191E-08	3.92161E-05
hsd17b7.S	8.248276769	4.78882866	66.83648729	2.94987E-16	3.00808E-12
snrnp40.S	8.024474355	1.598418485	30.67288619	3.05403E-08	8.49353E-05
skap2.L	7.888200093	2.575990896	23.28121141	1.39961E-06	0.003293596
fadd.S	-7.435468377	0.843525388	24.29868066	8.24955E-07	0.002103086
ND	-7.173340128	4.363419734	53.04578294	3.25864E-13	1.99377E-09
bet1l.S	6.89810175	2.893504438	67.98168104	1.65022E-16	2.52417E-12
tpst2.L	6.358451435	4.01028465	21.01545774	4.55593E-06	0.008710934
ND	5.598800827	5.736782308	19.70209486	9.05021E-06	0.012037566
hint1.S	5.252082735	4.111712812	20.7525723	5.22613E-06	0.009167286
dnajb2.L	-4.620672279	2.562191457	21.34584965	3.8345E-06	0.007827441
ipo11.S	-3.78250253	5.489666425	42.5128251	7.02203E-11	3.06883E-07
opn1lw.L	-3.191692101	0.947995856	17.87402749	2.3602E-05	0.025867682
pabpc1.S	2.948894926	5.614729356	20.69205616	5.39393E-06	0.009167286
ND	-2.538151977	6.564666314	41.44940417	1.20961E-10	4.62556E-07

Table 4 Differentially expressed genes: `DE_XEtreat` contrast. DE analysis between XE-E2 and XE-CON males (FDR < 0.06).

Gene symbol	logFC	logCPM	LR	PValue	FDR
ND	-17.43230386	1.127487298	28.77051259	8.14823E-08	0.000249271
ND	-17.43230386	1.127487298	28.77051259	8.14823E-08	0.000249271
ND	-11.61147061	2.294513489	21.40488298	3.71823E-06	0.009479008
ND	10.76547192	3.776122798	17.57555795	2.76115E-05	0.042234482
gtf2h4.S	-10.66477537	3.167980088	29.33074381	6.10199E-08	0.00023334
ND	9.778857922	2.226816916	18.3966469	1.79373E-05	0.030485498
pef1.L	9.223408055	3.868445671	18.7689547	1.4755E-05	0.028211468
snrnp40.S	-8.528167929	1.598418485	29.51010683	5.56259E-08	0.00023334
znrf1.S	-8.501163893	1.878632637	68.15097751	1.51444E-16	4.63299E-12
hsd17b7.S	-8.497715761	4.78882866	61.02864299	5.62504E-15	8.60407E-11
ND	-8.293920991	4.110252271	42.3380354	7.67846E-11	4.69799E-07
skap2.L	-7.976965294	2.575990896	20.22115076	6.8986E-06	0.01623399
ND	7.962385519	4.363419734	53.79654854	2.22363E-13	1.70063E-09
fadd.S	7.233822681	0.843525388	19.10454237	1.2375E-05	0.026948518
fam96a.L	-7.131684791	1.929950801	18.97943257	1.32135E-05	0.026948518
bet1l.S	-6.997857672	2.893504438	57.88529076	2.77857E-14	2.8334E-10
dnajb2.L	4.780682408	2.562191457	17.86276622	2.37421E-05	0.038227303
pabpc1.S	-3.288464231	5.614729356	18.57320288	1.63503E-05	0.029422819
ipo11.S	3.070766274	5.489666425	21.88743577	2.89121E-06	0.00804071
ND	2.791521094	6.564666314	35.67362228	2.33302E-09	1.18953E-05

Table 5 Differentially expressed genes: `DE_treat` contrast. DE analysis between SD-E2 and XE-E2 males (FDR < 0.06).

Annotation Cluster	**Enrichment Score: 1.3093279439095247**						
Category	*Term*	*Count*	*%*	*PValue*	*Genes*	*Fold Enrichment*	*FDR*
SP_PIR_KEYWORDS	nucleotide-binding	17	0.916936354	0.015924909	ACTA2, CAMK2G, ATP1A1, MCM6, SMC4, PDPK1, NME2, PSMC5, RND1, RAB18, PAK3, PIK3C3, RALB, PRKACA, TUBG1, RHOF, RAD17	1.830191728	16.9204725
GOTERM_MF_FAT	GO:0000166~nucleotide binding	23	1.240560949	0.038360098	ACTA2, CAMK2G, ATP1A1, ELAVL2, ACLY, MCM6, SMC4, PDPK1, NME2, HSP90B1, PSMC5, RND1, RAB18, PAK3, PIK3C3, RALB, CIRBP, PRKACA, RBM38, TUBG1, PARP1, RHOF, RAD17	1.48843457	37.70653517
GOTERM_MF_FAT	GO:0032555~purine ribonucleotide binding	19	1.024811219	0.043761002	ACTA2, CAMK2G, ATP1A1, ACLY, MCM6, SMC4, PDPK1, NME2, HSP90B1, PSMC5, RND1, RAB18, PAK3, PIK3C3, RALB, PRKACA, TUBG1, RHOF, RAD17	1.566510731	41.81053294
GOTERM_MF_FAT	GO:0032553~ribonucleotide binding	19	1.024811219	0.043761002	ACTA2, CAMK2G, ATP1A1, ACLY, MCM6, SMC4, PDPK1, NME2, HSP90B1, PSMC5, RND1, RAB18, PAK3, PIK3C3, RALB, PRKACA, TUBG1, RHOF, RAD17	1.566510731	41.81053294
GOTERM_MF_FAT	GO:0005524~ATP binding	15	0.809061489	0.04834439	ACTA2, CAMK2G, ATP1A1, ACLY, MCM6, SMC4, PDPK1, NME2, HSP90B1, PSMC5, RAB18, PAK3, PIK3C3, PRKACA, RAD17	1.692437066	45.09713549
GOTERM_MF_FAT	GO:0032559~adenyl ribonucleotide binding	15	0.809061489	0.049243972	ACTA2, CAMK2G, ATP1A1, ACLY, MCM6, SMC4, PDPK1, NME2, HSP90B1, PSMC5, RAB18, PAK3, PIK3C3, PRKACA, RAD17	1.688041126	45.72185044
SP_PIR_KEYWORDS	atp-binding	12	0.647249191	0.053136759	NME2, PDPK1, PSMC5, PAK3, ACTA2, CAMK2G, PIK3C3, ATP1A1, PRKACA, RAD17, MCM6, SMC4	1.832543927	46.76729281

Table 6 Functional annotation clustering of DE_pop genes. Above are the differentially expressed genes that show significant clustering by similar function (FDR < 0.50).

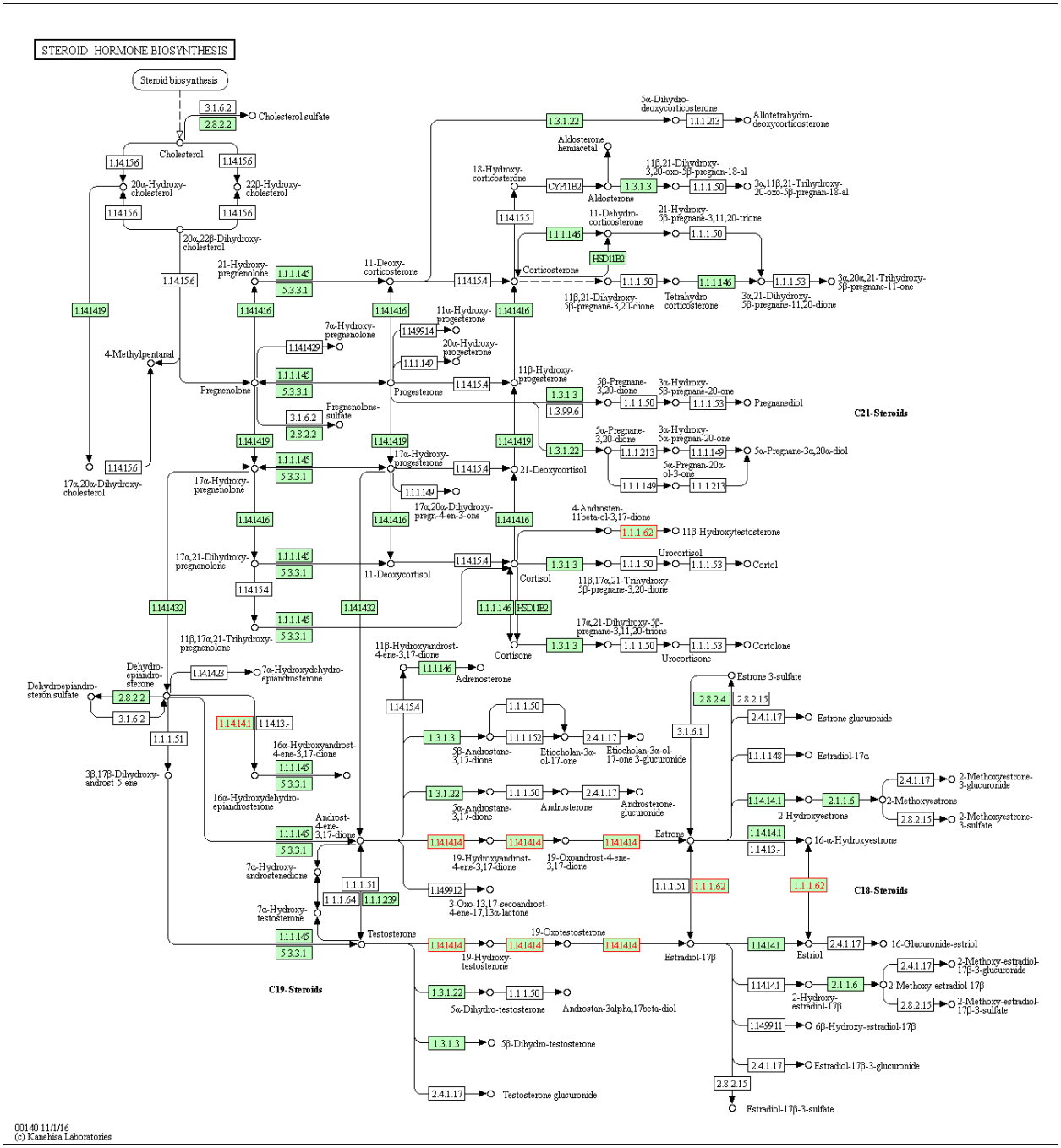
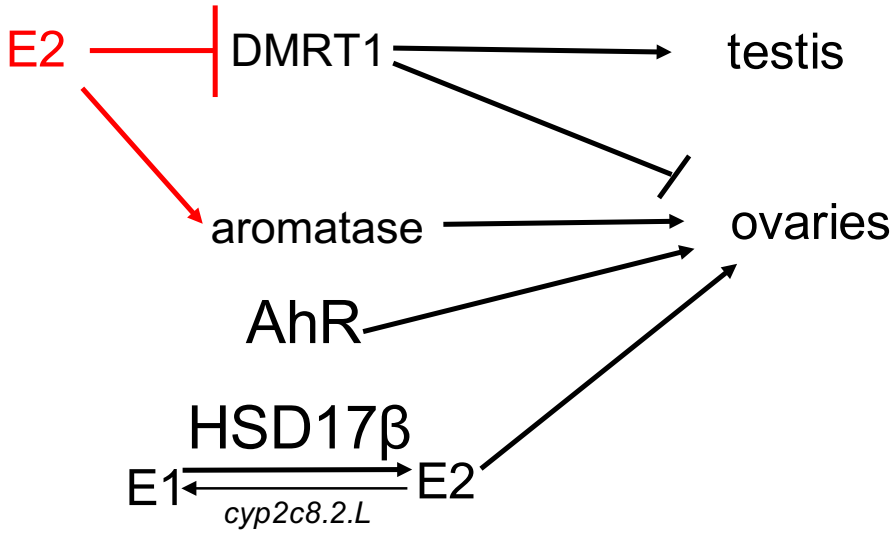
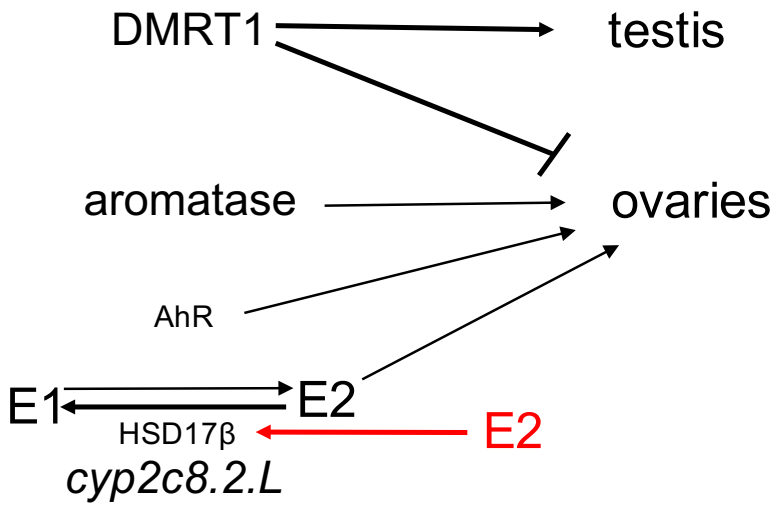


Figure 3 Steroid hormone biosynthesis in *Xenopus laevis*. Shown are all known pathways involved in steroid hormone production in African clawed frogs. Highlighted yellow and outlined in red are genes found to be differentially expressed between gonads of SD and XE tadpoles: *hsd17b7.S* (EC: 1.1.1.62), *cyp2c8.2.L* (EC: 1.14.14.1), and *cyp19a1* (EC: 1.14.14.14).



A. San Diego



B. Xenopus Express

Figure 4 Mechanism of population-dependent E2 susceptibility. (A) In the absence of E2, SD males were "pre-disposed" to feminization due to higher expression of ovary-supporting genes, *ahr.S* and *hsd17b7.S*, and lower expression of *cyp2c8.2.L*. After E2 exposure, SD males progressed further towards female development by downregulating a masculinizing gene (*dmrt1.s*) and increasing a feminizing gene (*cyp19a1*). (B) Decreased susceptibility of XE animals was associated with increased *hsd17b7.S* expression, which in this population, may have been metabolizing E2 to E1 in the presence on high exogenous E2.

Chapter Five

Conclusions

This dissertation elucidates the basis for differential responses to estrogen in populations of the African clawed frog (*Xenopus laevis*) and not only contributes to the field of developmental endocrinology, but may also hold great significance in environmental toxicology and animal conservation.

Chapter Two described a study that examined sex reversal rates between two source populations of *X. laevis*, Xenopus Express (XE) and San Diego (SD). In response to estradiol (E2) exposure, SD populations yielded greater numbers of morphological females than XE populations, and was thus defined as an E2-susceptible population and XE, an E2-resistant population. These findings were consistent for experiments using tadpoles from different parent pairs from each population and experiments testing familial replicates. Current literature addresses differences in susceptibility to E2 among different anuran taxa, but we are the first to describe differential E2 susceptibility between two populations of *X. laevis*, the model amphibian species. The characterization of these populations is valuable for researchers who may unwittingly be using *X. laevis* with extreme susceptibilities to test the effects of E2 or other forms of estrogen. Our results offer an explanation to the major inconsistencies in reported effects of estrogenic EDCs, which may stem from unaddressed differences in population E2 susceptibility. Notably, it would behoove scientists to study EDCs using the most vulnerable populations as they serve as sensitive bioindicators, while resistant populations may yield outcomes not representative of prevalent physiological effects.

We were motivated to examine whether this difference in response could be observed in another tissue at another life stage in *X. laevis*. Chapter Three tested the response of livers to E2 in adult XE and SD male frogs in order to investigate possible mechanisms that underlie variation in E2 susceptibility. This study was the only one to perform *in vivo*, immersion E2 treatment of male *X. laevis*, the exposure route most likely observed in natural conditions, with a wide range of concentrations (including physiologically relevant concentrations), and quantify and compare resulting *vtga2* and *esr1* mRNA synthesis, simultaneously, via

qRT-PCR between animals of two populations. SD males had higher plasma E2 levels than XE males for all E2 treatments. Exposure resulted in increased vitellogenin (*vtga2*) expression in both populations. However, SD animals had greater fold change in *vtga2* than XE animals at all E2 concentrations, while XE animals showed greater fold change in estrogen receptor (*esr1*) expression. These results confirmed that there are inherent differences in the susceptibility to the feminizing effects of E2, which can be observed in the population sex differentiation of the tadpole gonad and *vtga2* expression in the adult liver. Thus, the difference in E2 sensitivity spans tissue types and development stages. However, it is apparent that E2-response genes do not behave equally and researchers should be aware of these disparities when measuring genetic response to E2 exposure; *vtga2* and *esr1* expression may be an indicator of E2 exposure, but not necessarily an effective tool to directly measure the degree of exposure.

To fully understand the mechanism underlying E2 action during sex differentiation, we explored genome-wide analysis of gonad transcriptomes in ZZ-males. Chapter Four discussed the results of an RNA-Seq experiment examining differential gene expression between control and E2-treated groups of SD and XE populations. Although *X. laevis* is a model species, it is rarely used in genetics because it is allotetraploid, making genome assembly extremely difficult (Burgess, 2016). Until recently, its whole genome had not been completely sequenced (Session et al., 2016), resulting in a marked lack of transcriptomic studies of the species; in fact, there has only been one published study using the completed *X. laevis* genome in an RNA-Seq experiment (Ding et al., 2016). This current study, will be one of the first. Our analysis of differential expression showed different numbers of genes depending on statistical contrast. *ahr.S, cyp2c8.2.L*, *cyp19a1.L*, *dmrt1.S*, and *hsd17b7.S* were differentially expressed genes between the two populations that we recognized to have a role in sex differentiation and steroidogenesis. Based on the expression profiles, we proposed a mechanism underlying susceptibility to feminization, which involves both the suppression of masculinizing genes, the upregulation in feminizing genes, and changes in

E2 production and metabolism. In the absence of E2, SD males were "pre-disposed" to feminization due to higher expression of ovary-supporting genes, *ahr.S* (important in follicle development) and *hsd17b7.S* (E1 to E2 conversion), and lower expression of *cyp2c8.2.L* (E2 to E1 conversion). After E2 exposure, SD males progressed further towards female development by downregulating a masculinizing gene (*dmrt1.s*) and increasing a feminizing gene (*cyp19a1*). Decreased susceptibility of XE animals was associated with increased *hsd17b7.S* expression, which in this population, may have been metabolizing E2 to E1 in the presence on high exogenous E2. Together, these molecular players alter the ZZ-gonad's susceptibility to E2 and consequently, the sex-reversal rates of exposed populations.

The chapters in this dissertation present new evidence for population-specific response to E2 exposure that spans tissue types and developmental stages. The difference in sex reversal rates observed between XE and SD populations can be explained by the differential gene transcription detected via RNA-Seq. Furthermore, degree of susceptibility persists into adulthood and is consistent between gonad and liver. These data underscore the inherent differences in molecular and physiological responses of *X. laevis* populations and the need to elucidate the mechanisms behind these variations in order to conduct reliable experiments. It is crucial that researchers fully characterize and understand their animal model of choice. Further consideration must be taken when choosing populations or strains of a species as we have demonstrated vast disparities in their physiological response to E2.

Future studies will examine whether susceptibility is heredity and if it can be altered by breeding E2-susceptibile animals with E2-resistant animals. Experiments should be repeated with a variety of estrogens and estrogenic EDCs to test if these results are ligand-specific. Additional *in vitro* studies using isolated, cultured hepatocytes and gonads would allow for a more controlled test environment. This will also be an effective method to limit biological variation as cells from a single animal may be used for several treatments and as its own the control.

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