Environmental development of the external genitalia

by

Ciro Maurizio Amato

July, 2018

Director of Dissertation: Krista Ann-Marie McCoy

Major Department: Biology Interdisciplinary Doctoral Program in Biological Sciences

Hypospadias is one of the most common birth defects in the world. The incidence of hypospadias has nearly doubled over the past 50 years and now occurs on average in 1 in 125 male newborns each year. Hypospadias is a malformation of the penis where the urethra does not exit at the distal tip, but rather ventrally along the shaft of the penis. The severity of hypospadias depends on the amount of preputial swelling closure, the urethral exit, and the overall shape of the penis. The presence of anti-androgenic and estrogenic endocrine disrupting chemicals (EDCs) in the environment is strongly linked with hypospadias incidence and severity. Still, very little is known about the basic biology of penis development; how EDCs perturb normal development. Further, there are no known prenatal preventative treatments to protect the developing fetus from EDC exposure and abnormal penis development. In this dissertation I first reviewed the basic biology of penis development. Then using geometric morphometric analyses of developing genitalia I investigated the impact of the anti-androgen, vinclozolin, on morphological changes in the external genitalia. Based on the geometric morphometrics I then developed a quantitative scoring system to efficiently analyzed variation in penis development. The scoring system then was implemented to investigate the impact of EDC exposure of fetuses on hypospadias development and severity. Lastly, I used the basic biological information gained to investigate a potential prenatal preventative, sulforaphane, to protect the developing fetus from EDCs.

Environmental development of the external genitalia

A Dissertation
Presented To

The Faculty of the Interdisciplinary Doctoral Program in Biological Sciences

The Brody School of Medicine, East Carolina University

In Association with the Department of Biology, Thomas Harriot College of Arts and Sciences

In Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy

Interdisciplinary Doctoral Program in Biological Sciences

by

Ciro Maurizio

July, 2018

Environmental development of the external genitalia

by

Ciro Maurizio Amato

APPROVED BY:

DIRECTOR OF DISSERTATION: _____
Krista Ann-Marie McCoy, PhD

COMMITTEE MEMBER: _____
Jacques Robidoux, PhD

COMMITTEE MEMBER: _____
Elizabeth Ables, PhD

COMMITTEE MEMBER: _____
Xiaoping Pan, PhD

COMMITTEE MEMBER: _____
Edmund Stellwag, PhD

CHAIR OF THE DEPARTMENT OF BIOLOGY

_____
Jeff McKinnon, PhD

DEAN OF THE GRADUATE SCHOOL:

_____
Paul J. Gemperline, PhD

This dissertation is dedicated to Mr. K and Lil P for their unwavering support.

ACKNOWLEDGEMENTS

I acknowledge and thank Jenna Amato for her emotional support, jokes, critical feedback, and friendship. Jenna contributed many great ideas encompassed within this dissertation. I acknowledge my advisor Dr. Krista McCoy for passionately teaching me how to read, think, conduct, write, and present scientific research. I cherish the countless hours spent at the whiteboard. I thank Dr. Mike McCoy for providing a valuable, alternative perspective during joint lab meetings. I thank Josh Mogus for his extensive help on every project presented. Josh aided in animal care, dissections, tissue preparation, idea generation, and sanity maintenance. I acknowledge my committee members Dr. Jacques Robidoux, Dr. Elizabeth Ables, Dr. Edmund Stellwag, and Dr. Xiaoping Pan for their constructive feedback and suggestions throughout my graduate career. I thank the many undergraduate research assistants. There are too many for a comprehensive list, but I would like to acknowledge William Burton Burrows, Ariel Fricke, Sahiti Marella, Josh Yang, Morgan Boyd, Adam Baughm, Ross Cumoletti, and Fatoumata Diallo for their help in the lab, it was a joy to work with each of you. I would like to thank the office and housekeeping staff members in ECU's biology department for their support. I acknowledge Dr. April Blakesee, Rebecca Barnard, and Dr. Arianne Peralta for their help with DNA extractions and PCR in Chapter 5. I thank Tom Fink for his expertise in imaging and histology. I acknowledge the Ables lab for training and help with the Zeiss microscope in Chapter 4. I thank Bevin Blake for laboratory aid and critical comments on the technique generated in Chapter 1. I thank the biology graduate students from 2013-2018 for the priceless memories and support. It was a great ride. I would like to thank my mom for her unhindered confidence in me and for teaching me to follow my dreams. I thank my dad for instilling a hard work ethic and teaching me to be the best version of myself. Lastly, I thank my son for the joy he brings to me.

Table of Contents

LIST OF TABLES ..... *ix*
LIST OF FIGURES ..... *x*
Chapter 1: The morphogenic, hormonal, and gene expression changes controlling external genitalia development *1*
Abstract *1*
Introduction *1*
Sexual Dimorphic Morphogenesis of the Genitalia *3*
Sexually Dimorphic Hormonal Signaling in the Genitalia *5*
Androgen Dependent Development *6*
Estrogen Dependent Development *11*
Progesterone Signaling *15*
Morphogenetics downstream of hormone signaling *16*
Future Directions *20*
Acknowledgments *22*
Figures *22*
Chapter 2: Morphometric sexually dimorphic development is suppressed by Vinclozolin exposure *25*
Abstract *25*
Introduction *26*
Methods *28*
Animal care and use *28*
External genitalia imaging *29*
Analysis of Shape *30*
Statistical Analysis *30*
Results *31*
Major Axis of shape variation *31*
Allometric relationships change through time *31*
Discussion *33*
Vinclozolin exposure diminishes sexual dimorphism *34*
Utility for external genitalia shape analysis *35*
Acknowledgements *35*
Figures *36*
Tables *39*
Chapter 3: A validated protocol to quantify severity of male urogenital feminization using the MOUSE (Mouse Objective Urethral Evaluation) *41*
Abstract *41*
Background *41*
Methods *41*
Results *41*
Conclusion *42*
Introduction *42*
Results *44*
Discussion *46*

Advancing the mouse model of hypospadias *47*
Methods *49*
Mouse maintenance and treatment *49*
Generating variation in hypospadias severity *49*
Scoring hypospadias severity *50*
Validating scores *50*
Validation of our training protocol *52*
Statistical Analysis *54*
Acknowledgements *54*
Supplemental Methods *54*
Tables *56*
Figures *57*
Chapter 4: Organizational effects of the antiandrogen, Vinclozolin, on penis development in the mouse *63*
Abstract *63*
Introduction *64*
Material and Methods *66*
Mouse maintenance and treatment *66*
Does early anti-androgen exposure affect androgen receptor levels? *67*
Dose response *70*
Day vs. Dose Comparisons *70*
Histology and morphological data collection *72*
Statistical analysis *73*
Results *74*
Androgen receptor is diminished by early Vinclozolin exposure *74*
Early vinclozolin exposure potentiates continued antiandrogen exposure *74*
Discussion *77*
Understanding the causes of hypospadias severity *78*
Developmental-Endocrine signaling *79*
Relevance to Human Health *80*
Conclusion *81*
Acknowledgments *82*
Tables *87*
Figures *91*
Chapter 5: Mitigating EDC-induced hypospadias with the NFE2L2 activator, sulforaphane ... *98*
Abstract *98*
Introduction *98*
Results *101*
Sulforaphane protects the fetus from high antiandrogen exposure *101*
Sulforaphane reduces the Vinclozolin dose response *102*
NFE2L2 is required for sulforaphane-dependent rescue *103*
Discussion *104*
NFE2L2-mediated detoxification *106*
Sulforaphane reduces EDC-induced hypospadias in two strains of mice *106*
Non-monotonic responses to sulforaphane exposure *107*
Reducing hypospadias rates in the human population *107*

Material and Methods *109*
Mouse maintenance *109*
Chemical preparation *109*
Sulforaphane dose response with a single Vinclozolin dose *110*
NFE2L2 knockout study. *111*
Morphological data collection *111*
DNA extraction and PCR *111*
Statistical analysis *113*
Acknowledgements *114*
Figures *114*
Conclusions *117*
References *123*
Appendix: Animal Use Protocol *136*

LIST OF TABLES

Table 2. 1 Landmark order and description 39
Table 2. 2 P-values from MANOVA analysis 39

Table 3. 1Members of the public and graduate students can be quickly trained to score hypospadias severity 56

Table 4. 1 Calculated cumulative doses for each window 87

Table S4. 1 Details of antibodies used for immunohistochemistry 87
Table S4. 2 Weight and litter size mean and standard error for dose responses 88
Table S4. 3 Total pups analyzed for each treatment X dosing window regimen 89

LIST OF FIGURES

Figure 1. 1 Embryology of sexually dimorphic external genitalia development. 22
Figure 1. 2 Hormonal and genetic drivers of sexually dimorphic external genitalia development. 24

Figure 2. 1 Variation explained by relative warps. 36
Figure 2. 2 Majority of shape change occurs across developmental stage. 37
Figure 2. 3 Shape differentially scales with stage-dependent change in centroid size. 38
Figure 2. 4 Vinclozolin exposure reduces sexual dimorphism in external genitalia 38

Figure 3. 1Variation in hypospadias severity induced by vinclozolin. 57
Figure 3. 2Evaluating hypospadias severity. 58
Figure 3. 3 Scoring hypospadias severity. 59
Figure 3. 4 Scoring validation. 60

Figure S3. 1Hypospadias severity scores. 61

Figure 4. 1 Morphological landmarks for data collection 91
Figure 4. 2 Vinclozolin exposure on E13 and E14 diminishes AR expression. 92
Figure 4. 3 External morphology differs at the same doses. 92
Figure 4. 4 E13 Vinclozolin exposure induces higher rates and more severe hypospadias. 93
Figure 4. 5 Vinclozolin exposure on E13 causes more severe penile abnormalities. 94
Figure 4. 6 Vinclozolin exposure on E13.5 induces stronger effects. 96

Figure S4. 1 Negative Control. 97

Figure 5. 1 Sulforaphane rescues Vinclozolin-induced hypospadias and anogenital distance. 114
Figure 5. 2 Sulforaphane's rescue occurs across a Vinclozolin dose response. 115
Figure 5. 3 NFE2L2 is driving the sulforaphane-mediated rescue of hypospadias. 116

Chapter 1: The morphogenic, hormonal, and gene expression changes controlling external genitalia development

Abstract

The developing fetus receives and can respond to maternal, placental, and endogenous hormones, which all fluctuate in response to environmental conditions. These hormones organize fetal tissues so that they can respond to later hormonal signals and are competent to become sexually dimorphic at specific stages (times) in development. Many developmental studies, however, focus on the genetic mechanisms underlying morphogenesis and do not formally consider the upstream hormonal regulators that initiate sexually dimorphic development. For example, previous work describing genitalia development has focused on the role that androgen receptor and its downstream partners play, which has led to important discoveries of the genetic regulatory networks involved in genitalia masculinization. The research reported in this manuscript is meant to increase our understanding of the direct and indirect effects hormones exert on external genital development. This manuscript focuses on how hormone receptors are dimorphically expressed in male and female genital, how non-androgenic hormones affect genitalia development, and how hormone regulatory networks in the genitalia are affected by environmental chemicals. Here we review the literature related to the effects of hormones on external genitalia development especially in the context of male and female sexual differentiation.

Introduction

Endocrine signaling allows distant organ systems to communicate during development so as to coordinate cell and organ differentiation at the organismal level. Endocrine gland communication mechanisms generally involve secretion of hormones into the circulatory system where they travel to “target” cells and bind to specific hormone receptors. Endocrine regulatory

networks develop within the fetus or neonate and balance hormone signaling through feedback mechanisms that control circulating hormone concentrations. Feedback mechanisms are in place to either propagate developmental programs or maintain homeostasis (*1*). Alterations in hormone signaling and these feedback systems can ultimately impact developmental outcomes and have lasting effects into adulthood (*2*). For example, male and female external genitalia begin as seemingly identical bi-potential structures and subsequently develop into the penis or clitoris in males or females, respectively. The bi-potential genital precursor is known to be sensitive to a variety of circulating hormones especially androgens and estrogens which drive sexually dimorphic development of the genitalia. Alterations in sex steroid production, signaling efficacy, and regulation (e.g., feedback) can alter genitalia development (*3, 4*).

Most work describing genitalia development focuses on the gene expression profiles and genetic networks that occur within the genitalia downstream of androgen signaling. Although informative, this approach does not consider the milieu of hormones within which the fetus develops that ultimately regulate the developmental processes that define future structure and function. Understanding the diverse ways in which the endocrine system affects genitalia differentiation is innately interesting and is also medically important.

The number and severity of many congenital genitalia disorders is alarming; they are among the most common birth defects in humans (*5, 6*). To begin to understand the upstream hormonal signaling networks that induce development of sexually dimorphic external genitalia we need a synthetic understanding of the hormonal environments in which the genitalia develop. Past reviews have investigated the genetic networks that drive normal external genital development (*7, 8*), or the toxicological mechanisms through which environmental contaminants disrupt external genital development (*9, 10*). This review synthesizes the literature from a

different perspective and focuses on sexual dimorphic development, endocrine regulation, and steroid-dependent processes involved in regulating the development of sexually dimorphic external genitalia. Much of this review focuses on formation of the male genitalia, as the literature is strongly biased toward male development. We do discuss female genitalia development when the data is available and argue for more work to focus on the pathways leading to and resulting in full female genital differentiation.

Sexual Dimorphic Morphogenesis of the Genitalia

Genitalia development proceeds through two stages, at least from the perspective of steroid influence. The first stage is steroid independent and occurs prior to the differentiation of the gonads when the bipotential analgen of the genitalia forms within the tailgut region of the embryo (beginning at E9 in the mouse). The second stage is steroid dependent and drives sexual dimorphic morphogenesis ultimately leading to the development of the masculinized penis or feminized clitoris (from E13.5-puberty).

On E10.5 in mice there is no visible external genital structure, only an endodermal-derived cloacal membrane is visible in the tail gut region. (*11*). Beginning at E11-11.5 in mice, a pair genital swellings composed of mesenchymal cells appear externally and proximal to the endodermally derived cloacal membrane. This occurs as cells begin to aggregate or proliferate and form the bipotential genital tubercle (*11*) (Figure 1, E11-11.5). The external prominences continue to grow and fuse to a single genital tubercle, while the cloacal membrane covers the tubercle on the ventro-distal portion (Figure 1, E12.5-13.5). As the genital tubercle fuses and grows outward, dorsal mesenchyme from the umbilicus displaces the endodermal cloacal membrane, which then forms the presumptive urethral epithelium on the ventral aspect of the

genital tubercle (Figure 1, E12.5). At this stage the urethra is not tubularized distally and the cloacal membrane forms the urethral plate epithelium (*11*).

Development of the external portion of the genitalia described above occurs concomitantly with the growth of the internal urorectal septum mesenchyme. The urorectal septum originates from the allantoic ridge, which expands caudally to separate the cloaca into the urinary and alimentary canals (*11*) (Figure 1, E12.5-13.5). At the end of the androgen-independent portion of development, the urorectal septum fuses with the cloacal membrane to complete the septation of gastro-urinary systems (Figure 1, E13.5). The urinary tube that is formed partially extends into the external genitalia, referred to as the phallic urethra, and is connected to the developing bladder through the internal urethra (*12*). In the proximo-ventral portion of the genitalia the urethra exits through an opening described as the proximal urethral opening (PUO) or urethral duct (E13.5-16.5). The internal urethra does not extend to the distal tip of the external genitalia, instead the distal portion forms the urethral plate (*11*) (Figure 1, E14.5-16.5). As development progresses the urethral plate canalize to form an internal tube and then open on the ventral aspect of the tubercle. As development persists tubal closure leads to the formation of a seam along the ventral aspect of the tubercle (Figure 1, 17.5)(*12*).

On E14, externally, the preputial swellings composed of mesenchymal cells originate on the lateral portion of the genital tubercle. With continued growth, in both males and females, the preputial swellings grow toward and fuse at the ventral midline. They also grow distally. These preputial swellings become the prepuce of the penis or clitoris. Later in male development the preputial swellings fully envelope the glans penis. As the preputial swellings connect ventrally, the internal urorectal septum continues to extend to the tip of the penis (Figure 1 E17.5) (*12*). In males on E16.5 (Figure 1) the PUO (proximal urethral opening) is closed by the mesenchymal

infiltration. It is currently believed that as the urorectal septum moves into the penis, it pushes the internal urethra dorsally centralizing it and facilitating the formation of the distal meatus (*13*). The extent to which the mesenchyme of the preputial swellings infiltrates this region to help centralize the urethra is unknown and should be investigated further.

As the urorectal septum continues to grow in males the distance between the anus and base of the genitalia (anogenital distance, AGD) increases (Figure 1 E15.5-17.5). AGD is widely used in clinical research to estimate fetal masculinization (*14, 15*). Abnormally shortened AGD in males generally presents with a hypospadias and/or micropenis (*16*). In normal external genitalia development, the AGD becomes longer as the urorectal septum migrates further into the penis. Androgen signaling is known to increase the AGD, but research has yet to investigate the mechanisms downstream of AR that are responsible for increasing AGD.

In females, preputial swelling growth and urethral elongation are reduced compared to males. The urorectal septum remains near the base to separate the anus from the genitalia but does not infiltrate the genitalia to septate the urethral epithelium as in males. Instead, the urethra exits ventrally on the shortened genitalia, and females have a reduced AGD relative to males. In females the PUO also should be fully closed on E16.5-17.5 (Figure 1) and in postnatal development the PUO differentiates to form the vagina (*17*). In addition, the preputial swellings experience less ventral growth and do not envelope the tip of the glans. Through development morophological differences become more apparent, which are largely influenced differences in hormonal signaling.

Sexually Dimorphic Hormonal Signaling in the Genitalia

The cells within the external genitalia must be primed to appropriately respond to circulating hormones before they can undergo the sex hormone dependent morphogenesis which

results in sexually dimorphic development (Figure 2). Although, these signaling networks are established as early as E13.5 (*18*), the genitalia do not become morphologically sexually dimorphic until E16.5 or E17.5 in mice and rats, respectively (*19*). From E13.5-15.5 the penis is organized to respond to androgen signaling and undergo morphological change (*18*).The early expression of specific hormone receptors, and steroidogenic proteins facilitate expression and activity of downstream developmental genes that ensure proper morphogenesis.

In addition to the direct sex hormone induced changes in morphogenesis there are sex specific developmental programs that help suppress signaling required for genital development of the opposite sex (Figure 2). The mechanisms that program the genital tissues to become responsive to hormones and initiate and propagate (or suppress) differentiation are only beginning to be studied (*18*), but we do have a relatively well-developed understanding of what occurs once the tissues achieve hormonal responsivity.

Androgen Dependent Development

Since the 1970's it has been well established that normal penis development is dependent on androgen signaling (*20*). Jost showed that removal of embryonic testis resulted in female typic development. Diminished androgen signaling results in a demasculinized penis morphology, and excessive androgen signaling in females results in a hypertrophic, masculinzed clitoris. Several antiandrogenic and estrogenic chemicals are known to disrupt penis development, but the effects on clitoris development are less well studied.

On E9-E10 in mice, the gonads begin to differentiate into testis or ovaries largely through the activation of SRY in males and FOXL2 in females (*21, 22*). On E13.0 the fetal Leydig cells, which are the steroidogenic cell type in the fetal testis, convert cholesterol to androstendione (*23*). However, unlike the adult Leydig cells, fetal Leydig cells do not produce the enzyme that

converts androstendione to testosterone, CYP17A1 (*24*). The sertoli cells are responsible for the fetal conversion of androstendione to testosterone, which is then released into the bloodstream to induce signaling in distant tissues (i.e. external genitalia) (*23*).

As early as E11.5 androgen receptor mRNA can be detected in male and female bipotential genital tubercles (*25*). Unfortunately, the genetic programs controlling the initiation of androgen receptor transcription are not known. On E13.5 in mice, androgen receptor protein is present in the urethral epithelium in both male and female genitalia (*25*). The steroidogenic enzyme 5α-reductase, which catalyzes the hydroxylation of testosterone into its more potent metabolite, dihydrotestosterone is also present in both males and females on E13.5. Therefore, if exposed to testosterone, both sexes presumably can respond. On E14.5, however, 5α-reductase has become sexually dimorphic with females expressing much less protein within the preputial mesenchyme (*26*) relative to males. Whether expression of 5α-reductase is directly dependent on androgen signaling is currently unknown. On E17.5 in rats, mRNA for 5α-reductase isozyme 1 is strongly expressed in epithelial tissue, while isozyme 2 mRNA is strongly expressed in the mesenchymal cells in the penis (*27*). High levels of 5α-reductase mRNA expression strongly overlaps the expression of androgen receptor protein in mesenchymal cells within males (*26*). The different isoforms of 5α-reductase are known to have different expression patterns in other tissues and have different affinities for testosterone (*28*). Whether the different isoforms in the external genitalia function redundantly or provide some type of cell specific regulation is unknown.

Female rats exposed to testosterone during the masculinization phase of development (E 15.5-18.5) are defeminized (*29*). Androgen exposed females have more mesenchymal cells that infiltrate into the external genitalia, elongated urethras, and larger AGDs (*30, 31*). Congenital

adrenal hyperplasia (CAH) is the main cause of androgenization in human female external genital development (*32*). Approximately 1:10,000 cases of CAH are reported each year and 95% of cases are due to a 21α hydroxylase deficiency in the steroidogenic pathway. 21α hydroxylase is responsible for the conversion of progesterone and 17α -hydroxyprogesterone to deoxycorticosterone and 11-deoxycortisol, respectively (*33*). In 21α hydroxlase deficient patients the progestins are converted into androstendione and testosterone rather than into the corticosteroids (*34*). The elevated circulating adrenal androgens result in cliteromegaly and ambiguous genitalia in more severe cases (*34*). Allelic variants with differences in 21α hydroxlase activity show that the fetal female genitalia are dose dependently responsive to androgens(*35*).

As the external genitalia develops in an androgen rich environment, it expresses more androgen receptor and other androgen-specific factors for development (*18, 25*) likely due to positive feedback (*18*). Females exposed to testosterone on E13.5-16.5 show an upregulation in androgen receptor concentrations on E16.5 (*25*). In normal female development, the quantity of androgen receptor substantially diminishes as development proceeds, similar to the effects observed in male mice deficient in testosterone production (*25*). The Wolffian ducts in the male reproductive systems show similar responses to reduced androgen exposure during development. Mice exposed to flutamide (an androgen receptor antagonist) from E11-20 had much less androgen receptor expression in the mesenchyme of the Wolffian ducts than unexposed males (*36*). Importantly, this is the opposite pattern that we would expect in adults where androgen antagonism is expected to increase androgen receptors and testosterone due to a reduction in negative feedback (*37, 38*). This suggests that the androgen receptor is differentially regulated depending on the development stage of the organism.

Recent studies suggest that positive feedback mechanisms might be very important during sexually dimorphic development (*18*). For example, EDCs linuron (an androgen receptor antagonist) and Di-butyl phthalates (steroidogenic inhibitors) also reduce androgen receptor expression in the mouse Wolffian ducts (*39, 40*) further supporting the hypothesis that receptor-ligand binding increases androgen receptor quantity in the developing Wolffian ducts. Females exposed to dihydrotestosterone at the same development stage show an upregulation of androgen receptor in the Wolffian ducts (*36*). The positive feedback of androgen receptor is likely essential for normal penis development (*41*), but the mechanisms responsible for the feedback are not known and require further investigation.

Male mice exposed to the anti-androgen flutamide or vinclozolin, within the developmental period that external genitalia become sexually dimorphic, have varied levels of hypospadias incidence (*42-45*). Male mice exposed to high concentrations of hydroxyflutamide for two day intervals staggered throughout the hormone dependent phase, which begins on E13.5, experienced the highest hypospadias incidence when dosed on E15.5-16.5 (*46*). A similar sensitive period is observed with the 5α-reductase inhibitor, finestride (*26, 47*). Although when embryonic male mice are exposed E13.5 and E14.5 to the model anti-androgen vinclozolin, androgen receptor concentrations are significantly reduced which makes the genitalia more sensitive to antiandrogen exposure during the critical period (E15.5 and E16.5)(*18*). Early androgen signaling, therefore, likely sensitizes the cells of the presumptive penis to respond to later androgen signaling during the critical time period of sexual dimorphic development (*18*).

Epithelial androgen receptor knockout mice develop a normal penis with no distinct differences from wild type males. These results suggest that urethral epithelial androgen receptors do not significantly influence penis morphogenesis (*48*). Functionality assays on the

epithelial androgen receptor knockout have not been conducted, however, so it remains to be determined if epithelial AR plays functional roles independent of morphological development. Mesenchymal androgen receptor knockouts display defects in urethral tubulerization and urorectal septum growth (*48*). Indeed, a number of studies show androgen receptor to be strongly expressed in the mesenchyme of the leading edge of the preputial swellings as they form the ventral seam of the penis (*18, 25, 48-50*). Although androgen receptor is a major factor responsible for initiating masculinization, there are important regulatory factors involved in facilitating androgen signaling or inhibiting estrogen signaling.

Fkbp52 is a chaperone protein that facilitates proper androgen signaling in the genitalia. Fkbp52 alters the folding of androgen receptor and increases its affinity for circulating androgens (*51*). Fkbp52 is found in the urethral epithelium and throughout the preputial mesenchyme, completely overlapping with androgen receptor rich regions (*52*). Loss of Fkbp52 strongly demasculinizes the male genitalia and reduces the migration of the urorectal septum due to the reduced affinity of the androgen receptor to circulating androgens (*52*). Interestingly normal penis development of Fkbp52 mutants could be partially rescued when pregnant dams were supplemented with testosterone, supporting the hypothesis that Fkbp52 is important for making androgen receptors more responsive to circulating androgens (*52*). Sensitization of androgen receptors by chaperone proteins or other uninvestigated mechanisms (e.g., phosphorylation and co-activators) could be especially important when other circulating hormones can bind to cognate receptors which is expected to negatively impact androgenic signals. Indeed, the mechanisms controlling the potency of androgens within the genitalia during development are also unknown.

Androgen signaling is known to upregulate the expression of cytochrome P450 1B1 (CYP1B1) in the external genitalia. The role of CYP1B1 in the external genitalia has not been defined but it is likely involved in metabolizing estrogen into a more inert form (*53*). CYP1B1 is localized in the preputial and dorsal mesenchyme of the genitalia and is expressed more in males than females. CYP1B1 is shown to be involved in angiogenesis, which is essential for male growth and vascularization of the external genitalia. Previous studies on carcinogenesis have shown that CYP1B1 preferentially metabolizes 17ß-estradiol to a more inert or even anti-estrogenic catechol estrogens (*54, 55*). CYP1B1 could, therefore, function in males to prevent aberrant estrogen signaling potentially induced by basal circulating levels from the mother or basal metabolism of testosterone into estrogen. CYP1B1 might become particularly important in ensuring proper penis development, when the fetus is exposed to environmental estrogens. Interestingly however, CYP1B1 has also been shown to hydroxylate testosterone to a more inert hormone(*56*), which could either help reduce excessive testosterone binding or reduce the testosterone to estrogen conversion. Appropriate androgen and estrogen signaling controls expression patterns of a suite of genes important for development (*48, 50, 57, 58*) (Figure 2). Reduced androgen signaling in males results in female typical protein and gene expression patterns, and thus leads to altered penis development.

Estrogen Dependent Development

The importance of estrogen signaling in external genital development has been fairly under studied. Based on Jost's previous experimental research, female external genital development has been described to occur in the absence of hormone signaling (*59-61*). Several studies, however, suggest that estrogen signaling is required for normal female development (*presented below (25, 62-65)*). The majority of information presented henceforth is mostly

related to estrogen's role in altering male development because its role in female genital development is understudied and available information is presented below.

Similar to androgen receptor, estrogen receptor is expressed by E13.5 in the mouse at similar levels in both males and females. Estrogen receptor has three main isoforms, estrogen receptor-α, estrogen receptor-ß, and estrogen receptor-γ (*66*). The three separate estrogen receptors allow for increased tissue specificity and a diverse array of tissue specific effects induced by estrogen exposure (*67*). Estrogen receptor-α and ß have highly conserved DNA binding domains suggesting they are binding to similar cis-regulatory elements, but the N-terminal domain between the two are much less conserved, which suggests different suites of co-regulators can act on each receptor (*68*). In normal female development, the amount of estrogen receptor increases across time (E13.5-16.5) at similar rates as androgen receptor in males (*25*). Both mRNA and protein levels of estrogen receptor-α showed time dependent increases in expression in fetal female mice (*64*). Estrogen receptor-ß expression stays approximately the same throughout genitalia development in females and slightly decreases across time in males (*64*). As with androgen receptor, the mechanisms responsible for the initial expression of estrogen receptors and their increased expression through time are unknown. Estrogen receptor-α knockout resulted in a 59% larger clitoris and the presence of an enlarged bacculum and cartilage in the external genitalia in adult female mice (*65*), indicative that estrogen receptor-α plays a role in external genitalia development.

Prenatal exposure to 17ethinyl-estrogens induce hypospadias and other abnormalities in male development (*63, 69*). The estrogen-induced changes in masculinization and penis development, could occur directly via estrogen receptor signaling or indirectly if estrogen exposure reduces testosterone production in the male fetus. The mechanisms responsible for this

effect have not been thoroughly investigated likely because estrogens have been dismissed for years as being important in genital development.

In the rat penis, high concentrations of aromatase are found within the first week after birth and precipitously drops afterward (*70*). Diethylstilbesterol, testosterone, and dihydrotestosterone all upregulate aromatase expression in the postnatal penis (*70*). Therefore, both endocrine disrupting chemicals and endogenous hormones could alter estrogen signaling in the genitalia of newborn boys. Over expression of estrogen responsive genes has been identified in feminized male (human) genitalia throughout fetal development and at postnatal periods suggesting that altered estrogen signaling induces feminized genitalia. However, we do not understand the mechanisms though which aberrant postnatal estrogen signaling affects genital development or adult function in humans.

Another mechanism through which estrogens can affect genital development is via upregulating activating transcription factor 3 (ATF3). In humans, gene polymorphisms and aberrantly increased gene copy number of ATF3(*71, 72*), a cAMP responsive element binding (CREB) protein, induces hypospadias in males. ATF3 is estrogen responsive, and interacts with the androgen receptor as a co-repressor, which decreases transcriptional activity of the androgen receptor (*73*). ATF3 increases in the genital tubercle mesenchyme from E12-newborn mice and is higher in female mice compared to males on E19 (*74*). Presumptive male mice exposed to ethinyl-estradiol from E12-17 and examined on E19 have higher expression of ATF3 mRNA relative to control males (*74*). During normal development E12-17 male and female external genitalia do not differ in ATF3 expression (*74*). Later in development, after exposure ceased, (E19) differences in ATF3 expression were detected, suggesting that early estrogen exposure

alters future ATF3 expression in the external genitalia which is expected to decrease androgen signaling.

An estrogen receptor co-regulator, vesicle associated membrane protein 7 (VAMP7), is associated with reduced anogenital distance, and penis length, and hypospadias incidence in humans (*75*). In addition, transgenic male mice expressing more VAMP7 have no change in testosterone levels, but have more cases of hypospadias, reduced AGD, and reduced penis length. VAMP7 colocalized with estrogen receptor-α in the nucleus and intensified transcriptional rates by the estrogen receptor (*75*). The elevated estrogen signaling in VAMP7 transgenic mice resulted in more ATF3 expression and other estrogen responsive genes (*75*). As mentioned above ATF3 reduces androgen signaling. Taken together, estrogens are likely modulating the amount of androgen signaling during female genital development which prevents aberrant masculinization of the female reproductive organs. Indeed, females need androgens to produce estrogens, and must carefully control androgen signaling. Therefore, evolving mechanisms to reduce androgen signaling (e.g., AR quantity and function) would be strongly selected for to prevent aberrant masculinization of the female genitalia, especially if fetal tissues are regulated by positive feedback.

Most laboratory research on estrogen exposure in relation to male external genitalia has been conducted postnatally. When mice are dosed with the synthetic estrogen, Diethystilbesterol, during postnatal development occurring between P0-13 they exhibit a micropenis phenotype as adults (*76*). Anti-androgen exposure during this time period does not induce micropenis (*44*), therefore the estrogenic effects are likely not induced by reduced testosterone production that might be expected to occur with increases in estrogen exposure. Indeed, daily exposure to estrogen for four days in estrogen receptor-α knock out models does not show the same

micropenis phenotype as wild type exposed mice (*77*). Although the estrogen receptor-α knockout males are infertile due to testicular abnormalities, they have normal penis development, as opposed to the wild type males exposed to estrogen that have reduced penile length and weight (*77*). This means that, in mice, the penis continues to develop postnatally and it is responsive to estrogens which demasculinize penis development.

In sum, estrogens are likely playing multiple roles in the developing external genitalia. Estrogens might prevent basal androgen signaling to ensure proper female genital development. For example, ATF3 and VAMP7 interact with both the estrogen and androgen receptor to increase estrogen signaling and reduce androgen signaling (*75*). Estrogens likely directly alter normal penis development via estrogen receptor and could also play an important role in clitoral development by reducing growth of the genital tubercle. Further research on the female development and the potential antagonistic relationship between androgen and estrogen signaling is necessary for developing a thorough understanding sexual dimorphic development of the genitalia. Determining circulating and tissue concentrations of androgens and estrogens during development will aid in our understanding of how the external genitalia is being influenced by normal and disrupted hormone signaling.

Progesterone Signaling

Progesterone signaling has been significantly less studied in the context of external genitalia development compared to androgens and estrogens. Circulating progesterone has been detected in the developing fetus, so it is possible that progesterone could influence genital development. The time scale over which progesterone receptor is expressed in the genitalia is not well characterized, but within the genitalia of CD1 mice it is significantly lower in males than females on E19.5 (*78*).

Exposure to medroxyprogesterone, a progesterone receptor agonist, from E12-17 resulted in increased progesterone receptor, estrogen receptor, and androgen receptor in the genitalia of males, but decreased mRNA expression levels for all three receptors in the females (*78*). This sexually dimorphic expression suggests that progesterone plays an important and sex-specific roll in defining the diversity and quantity of steroid receptors in the developing genitalia. The exposure to medroxyprogesterone also induced malformations in both male and female external genital development. Males had a more proximally exiting (demasculinized/feminized) urethra, while females had a more distally exiting (masculinized, defeminized) urethral exit (*78*) suggesting that the balance between signaling molecules has important developmental consequences. Now, as to whether the effects observed were caused by the differential concentrations of androgens and estrogens, or due to downstream targets of progesterone, or binding of medroxyoprogesterone to alternate receptors is unclear. The work that has been accomplished on progesterone's role in genital has demonstrated that development is dependent on a delicate balance in the concentration and timing of steroid signaling which influence other hormonal signaling pathways.

Morphogenetics downstream of hormone signaling

Hormone signaling plays an important role in the divergence in downstream gene expression that controls developmental outcomes. Here we synthesize the literature that determines how hormones influence expression of some of the main developmental genes involved in sexual dimorphic morphogenesis.

Fibroblast Growth Factor Receptor 2 (Fgfr2IIIB) is upregulated by androgen signaling and is strongly expressed in the prepuce and within the urethral plate epithelium in male mice on E16.5 (*50, 57*). Indeed, anti-androgen exposure reduces Fgfr2IIIB expression in the urethral

epithelium (*50*), which results in development of hypospadias and diminished growth and closure of the preputial swellings. The ligand for Fgfr2IIIB, Fgf10 is predominately expressed in the distal urethral epithelium and signals through paracrine signaling to activate Fgf signaling in neighboring cells and trigger expansion of the preputial swellings and maturation of the urethra(*79*). Global knockouts of Fgfr2IIIB or Fgf10 results in severely diminished preputial swelling growth. These knockouts do not resemble the wildtype female phenotype, but rather are completely missing external genitalia or are grossly malformed. This result suggests that Fgf signaling is generally important for genitalia development but not for sexual dimorphism per se. Studies investigating endodermal and ectodermal specific Fgfr2 knockouts show that endodermal Fgfr2 is required for maturation of the urethral epithelium and that ectodermal Fgfr2 is required for preputial development and urethral development in males (*80*). Female endodermal Fgfr2 knockouts show few changes when compared to the wildtype, but ectodermal Fgfr2 knockout show severe preputial dysgenesis similar to that in male ectodermal Fgfr2 knockouts (*80*). Fgfr2 is required for both female and male development, but may function differently under androgen or estrogen signaling.

Fgf signaling is tightly linked with sonic hedgehog signaling (Shh), and this interaction between Fgf and Shh is necessary across several developmental systems for polarizing activity and proper growth. Shh signaling in the developing genitalia from E11.5-13.5 regulates cell cycle kinetics by shortening the G1 phase thus increasing proliferation in the developing genitalia (*81*). Shh expression occurs in the endodermally derived urethral epithelia (*50*) in both male and female genitalia and is essential for genital outgrowth in both sexes. For example, removal of Shh from E11.5-13.5 results in persistent cloaca and no external genitalia (*81*). Fgf acts to upregulate Shh expression in the urethral epithelium throughout external genitalia

development. Removal of Shh signaling after initiation of genital outgrowth and during the critical time when androgen signaling is driving sexual dimorphism of the genitalia (E13.5-15.5), in male mice, results in a similar phenotype to that observed in FGF knockouts (*57*). There is little preputial swelling outgrowth and the urethra exits ventrally along the shaft of the penis. Females also have abnormally formed genitalia when Shh is removed during this same time period (E13-17), and both sexes show a reduction of cell proliferation in the urorectal septum (*57*). Even in early development removal of Fgf signaling reduces Shh signaling in the urethral epithelium, and also leads to loss of genital development at E10.5. Removal of Shh in the urethral epithelium reduces Fgf expression in the distal epithelium, which leads to the lack of external genitalia development (*82*). Therefore, both Fgf and Shh feedback on one another and regulate each other's expression levels. These data show that Shh is required throughout development and tightly interacts with Fgf to promote proliferation and differentiation of mesenchyme and epithelium in the genitalia to achieve proper male and female development.

Shh also interacts with the Wingless-Type MMTV Integration site (Wnt) signaling pathway. Shh removal reduces Wnt expression in the epithelium (*82*). When Wnt is bound to its receptor, Frizzled, ß-catenin is activated and interacts with Lymphoid Enhance Binding Factor (LEF) transcription factors to induce transcription of Wnt-dependent genes (*83*). Differences in apoptosis or proliferation have not been recorded in Wnt/ß-catenin knockouts, but Wnt has been associated with cellular migration. During development, male genitalia have higher levels of Wnt and ß-catenin expression than females (*48*). Males with ß-catenin knocked out in the preputial mesenchyme have a ventrally exiting urethra and hypospadias, and females with over expressed ß-catenin have a more masculine phenotype (*48*). Females actually express a potent inhibitor of Wnt, Dickkopf-2 (Dkk2) (*48*). On E17.5 if females are exposed to testosterone they

downregulate Dkk and upregulate ß-catenin expression whereas flutamide (an AR antagonist) exposed males have female-like upregulated levels of Dkk2 (*48*). This research shows that Wnt/ß-catenin signaling and its associated masculinization can be controlled by androgens via Dkk2. Recently, however, it was shown that males exposed to estrogen, but not antiandrogens, have elevated Dkk2 expression in the mesenchyme (*25*). Although relatively little work has been conducted to determine the effects of estrogens on genitalia development this work on Dkk2 suggests that a balance between estrogen and androgen signaling drive genitalia development in both males and females.

Indian Hedgehog (Ihh) is part of the hedgehog family and is involved in epithelium to mesenchyme signaling as well as in cellular differentiation and proliferation (*84*). Males exposed to either flutamide or estradiol benzoate had 3 or 11-fold reductions in Ihh expression intensity, respectively (*25*). The impacts of IHH on external genital development remains unknown. Interestingly, epithelial expression of Ihh in the uterus negatively regulates estrogen receptor expression and estrogen signaling within the stromal tissue, which aids in embryo implantation (*85*). Thus, Ihh is negatively regulating estrogen signaling within the uterus. Although the penis and uterus are not analogus structures, the regulation of IHH may remain conserved and may function the same way in the two structures. Whether Ihh is transcribed by ER or AR is not known, but a similar Ihh-mediated estrogen receptor repression could be observed within the developing external genitalia further suggesting a role for estrogens in normal genital development.

Musculoaponeurotic fibrosarcoma oncogene homolog B (MafB) is an androgen dependent transcription factor that contributes to cell differentiation and organogenesis in other organs (*86*). MafB is expressed at similar concentrations in males and females at E13.5, but then

expression increases at E14.5 in males (*49*). Female MafB expression remains very low at E14.5, but dihydrotestosterone exposure increases MafB expression to male-typical levels (*87*). MafB is localized at the leading edge of the preputial swellings and strongly co-localizes with androgen receptor expression (*49*). MafB knockout males display diminished preputial swelling outgrowth and failure of urethral closure (*49*). Indeed, androgen receptor directly regulates MafB transcription by binding to an androgen response element in the 3'-UTR in the MafB gene (*87*). Taken together, these results suggest that that is an interplay between androgen and estrogen signaling that directly drive the differentiation of the external genitalia.

Future Directions

External genitalia defects are very common when compared to other congenital defects, but the effects of hormone action remain poorly characterized. Researchers have begun to understand the genetic regulatory networks involved in maintaining external genitalia development. We are, however, only beginning to characterize the endocrine regulatory networks that control these genetic regulatory networks to regulate genital development. Early in development, prior to any morphological change, the external genitalia are being primed by circulating hormones (*18*). These hormones upregulate or downregulate steroid receptors and steroidogenic proteins (e.g., 5α reductase, cyp1b1, aromatase). The patterning and regulation of these proteins sensitize or condition cells in the genital tissue primordia to respond to later signals well before morphological change begins (*18*) (Figure 2). Whether androgen and estrogen signaling are directly or indirectly antagonizing one another, and how their interactions are influences by hormone interactions remain unknown. However, are questions that are left unanswered.

The endocrine system serves to integrate different organs systems to allow communication, especially during fetal development when the fetus depends on maternally and placentally derived hormonal signals as well as those originating from its own, non-placental endocrine system (*88, 89*). The roles that the mother and placenta play in the upstream endocrine signaling that control genitalia development are unknown. Also, few studies address ovary steroidogenesis and development in rodents. Some studies have classified the ovaries as hormonally active. In rats aromatase, which converts testosterone to estradiol, is present in the fetal ovary and cAMP activates estrone and estradiol production as early as E14 (*90, 91*). More research should address the steroidogenic capacity of the ovary and its importance in external genitalia development. Furthermore, few studies have investigated development of the negative and positive endocrine feedback mechanisms that control endocrine signaling in the fetus. Indeed, the development of endocrine regulation (e.g., feedback mechanisms), organization of the external genitalia so that it can respond to endocrine signaling, and the role of the balance between hormone signals (e.g., testosterone to estrogen ratios) have not been studied in detail and require further investigation. Using a combination of systems level approaches, and targeted hypothesis driven research to identify the upstream endocrine regulatory networks will help us begin to understand the etiology of congenital defects induced by endocrine disrupting chemicals.

When moving forward to define the endocrine regulatory mechanisms orchestrating external genitalia development and other sexually dimorphic structures, we must more carefully and formally consider several concepts that have as of yet remained understudied. First, we need to better understand how endocrinological negative and positive feedback mechanisms develop across organ systems (i.e., within the Hypothalamus-pituitary-gonad axis), and within the target

tissues themselves. Second, we must attempt to understand the mechanisms through which sexually dimorphic cells are primed and are made responsive to hormones that will be activated during specific windows of development. As this process is tissue specific each tissue can have its own non-monotonic responses to various hormones and EDCs (*18*). Although much attention has been given to the mechanisms downstream of AR that influence penis development, we have little information on what factors regulate early sex hormone production and organize tissues so that they can respond to those hormones when the hormones begin to be synthesized embryonically. We are hopeful that this review has inspired new ideas that will refocus the field. Gazing upstream is, in our opinion, just as wondrous as looking downstream.

Acknowledgments
We would like to acknowledge Josh Mogus and Daniel Newhouse for help formalizing the ideas discussed in the review.

Figures

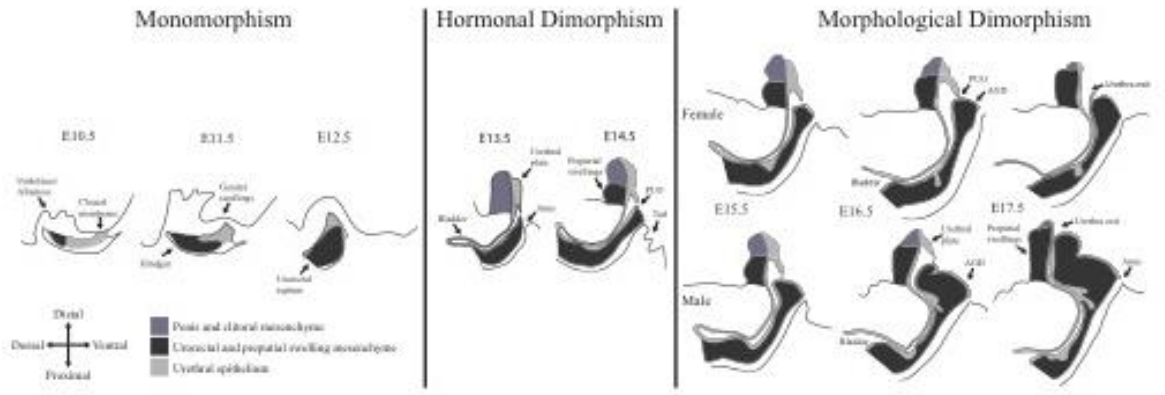


Figure 1. 1 Embryology of sexually dimorphic external genitalia development.

From E10.5-12.5 the external genitalia are developmentally identical for males and females. On E10.5 there is only a cloacal membrane between the tail and umbilicus. On E11.5 a pair of genital swellings begin to form and push the cloacal membrane onto the ventral aspect of the external genitalia by E12.5. While the external swellings form, internally the urorectal septum grows toward the genital swellings. As the septum grows, the urogenital and anorectal canals become separated. On E13.5-14.5 male and female external genitalia are morphologically similar, but hormonal and genital signaling are dimorphic. On E13.5 the urorectal septum fully separates the urogenital and anorectal canals. The genital swellings have grown and the cloacal endoderm covers the ventral aspect and is now referred to as the urethral plate. On E14.5 a pair of preputial swellings appear on the proximo-dorsal aspect of the developing genitalia. E15.5 marks the first day where subtle differences in morphology can be identified between what will become males and females. The main morphological difference is in the size of the region separating the anus from the genitalia. By E16.5 the AGD is drastically different between males and females, also in males there is mesenchymal infiltration into the external genitalia that begins to separate the urethra from the ventral aspect of the penis. By E17.5 the mesenchymal infiltration has extended near the tip of the penis and the urethra is positioned in the center of the penis, while in females the urethra exits ventrally and close to the base of the external genitalia.

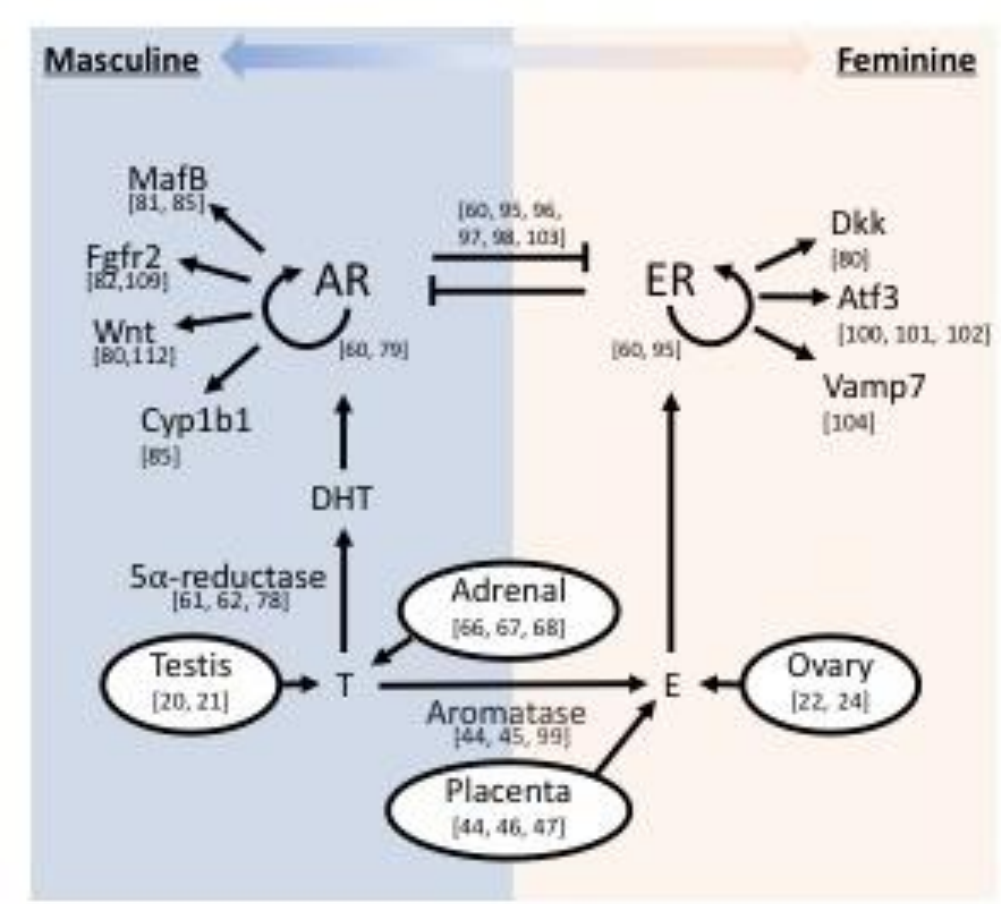


Figure 1. 2 Hormonal and genetic drivers of sexually dimorphic external genitalia development.

Early in development the external genitalia begin in an undifferentiated bipotential state. The external genitalia begin to diverge in morphology after sex steroid production begins largely in the gonads and to a lesser extent in the adrenal gland and placenta. Circulating androgen and estrogen then bind to androgen or estrogen receptors, respectively, and control 1) autoregulation of the respective receptor, 2) inhibition of the alternate receptor, and 3) expression of sex steroid dependent genes that specify the appropriate pathway of genital development.

Chapter 2: Morphometric sexually dimorphic development is suppressed by Vinclozolin exposure

Abstract

Structural defects remain among the most common birth defects in the United States, but the etiology of structural congenital defects remain largely undefined. Abnormalities of the external genitalia are common structural defects that present in boys and the hormonal, genetic, and environmental factors responsible for inducing alterations in penis morphology still remain largely unknown. The presence of endocrine disrupting chemicals (EDC) in the environment correlate strongly with the defects of the external genitalia. No quantitative studies have evaluated when the external genitalia normally become sexually dimorphic and when EDC exposure derails the sexually dimorphic developmental pathways that specify morphogenesis. To address this gap in our knowledge this study evaluates genital development through a developmental series by comparing control and vinclozolin exposed male and female mice at developmental stages beginning at E13.5 through E18.5. Genitalia of individuals collected over six days during development (E13.5-18.5) were analyzed using geometric morphometrics and the resulting data statistically analyzed with MANOVA. Data was visualized with estimates form canonical discriminant analysis. We found that on E13.5-14.5 the external genitalia are indistinguishable between sex and treatment. As early as E15.5 there were significant differences in the shape of the gentials that were related to treatment, sex and stage collected. Vinclozolin exposure reduced the degree of sexual dimorphism exhibited relative to the control animals. The normal sexual dimorphism increased across time such that control males and females were highly divergent at E18.5. The impacts of vinclozolin were still apparent on E18.5 even though vinclozolin treatment was terminated at E16.5. We conclude that vinclozolin has opposite impacts on male and female genital development, where males become feminized and females are masculinized in morphology. Also future studies investigating sexual dimorphic development should sample prior to E15.5.

Introduction

Proper differentiation of anatomical structures is essential for an organism's survival and reproduction. Structural birth defects are among the most common congenital disorders including those that affect the heart (*92*), genitalia (*6*), palate (*93*), and limbs (*94*), among others. These disorders occur throughout the population and induce a variety of disabilities. In addition to the genetic disorders (~20% of cases) that lead to structural birth defects, a number of persistent teratogens in the environment are known to impact development directly (~10% of cases) and affect genetically susceptible patients (~70% of cases) (*95*). During morphological development endocrine, paracrine, and autocrine signaling molecules are necessary to direct body plan organization and the defined establishment of specific morphological structures like the face and external genitalia (*96-98*). Perturbation of these signaling molecules causes miscommunication between cells that ultimately define these structures and thus causes abnormal development (*94, 99-102*). The increased prevalence and quantity of synthetic teratogenic chemicals in the environment is correlated with the severity and probability of humans developing structural birth defects (*103, 104*).

Birth defects of the external genitalia are one of the most common categories of congenital deformities in the world (*6, 105, 106*) Unfortunately, between the years of 1940-1990 the incidence of hypospadias, a penis abnormality, doubled in prevalence (*6*). Hypospadias is a malformation of the penis in which the urethral exit does not occur at the distal tip of the penis, but rather ventrally along the shaft of the penis (*107*). In addition to a ventrally exiting urethra, hypospadias can also coexist with other structural malformations of the penis. For example, penis torsion, clitoral hood, ventral curvature of the penis (chordee), micropenis, and blind vaginal openings all have been documented to co-occur with hypospadias (*108-110*). Severity of hypospadias and other penis defects vary considerably based on the genetic makeup of the individual and the environment in which the fetus develops (*111*).

The incidence of hypospadias and other external genitalia malformations are strongly correlated with occupational and environmental exposure to endocrine disrupting chemicals (EDCs) (*16, 112, 113*).

EDCs are a class of chemicals that modulate the endocrine system by agonizing or antagonizing hormonal signals (*25, 29, 41, 114, 115*). Prior to gonad differentiation the male and female external genitalia develop in a seemingly identical fashion (*116*). After differentiation of the testis or ovaries, increased amounts of sex steroids are found circulating in the blood stream (*117*). Penis development is largely dependent on androgen signaling, with androgen insensitive male gentialia resembling those of female external genitalia (*118*). A number of anti-androgenic and some estrogenic EDCs induce hypospadias across mammalian taxa, demasculinizing or feminizing penis development (*25, 29, 41, 114*). Development of the female external genitalia has been studied to a lesser extent, but it is largely thought that females develop normally in the lack of androgen signaling. However, one study does suggest that anti-androgen exposure may masculinze female development and points to a possible role of androgens in the specification of female genital development (*119*).

In the past, visual assessments of genitalia morphology and linear measures of urethral length and genitalia length have been used alone to compare treatments, genotypes, and sexes (*69, 119, 120*). This type of analysis is sufficient and appropriate for specific questions regarding urethral tubulogenesis or genital outgrowth but falls short when addressing broader questions on the hormonal, environmental, and genetic drivers of morphogenesis because it fails to incorporate gross morphological changes of the sample. Geometric morphometric analysis allows for an overall analysis of both shape, size, and allometric changes throughout development (*121-123*). Allometry is the differential scaling between shape and size of a structure (*124*). Often, as size changes shape also changes but not at the same rate. Here we use the model anti-androgenic EDC vinclozolin and the application of geomorphometric analysis, to investigate the impact of androgen receptor blockade on the size, shape, and allometric relationship of size and shape through development of male and female external genitalia.

Geometric morphometrics was used to analyze shape differences through developmental time. Investigation of shape differences allow us to determine if anti-androgen exposure impacts male and female development, where in the external genitalia the impacts are occurring, and when in

developmental time those changes arise. We hypothesize that vinclozolin exposure will alter male penis development to mirror the morphologies typically associated with female developing genital structures. Further, based on previous female exposures to anti-androgenic chemicals we expect female development to be masculinized by vinclozolin exposure.

Methods

Animal care and use

All experiments were approved by East Carolina University IACUC under animal use protocol D-297 and D-297a. CD1 mice were purchased from Charles River (Raleigh, NC) at 7 weeks of age. Mice were fed ISOPURE rodent chow and housed on 12 hr light/dark cycles. Upon arrival at ECU the mice were acclimated to the housing facility for one week. After acclimation mice were time-mated with the presence of a vaginal plug recorded as embryonic day (E) 0.5. After a vaginal plug was observed, mice were ear tagged and jointly housed until treatment. Pregnant dams (n=3-5) were randomly assigned to a corn oil control or vinclozolin treatment and were assigned to be collected at one of six developmental stages (E13.5, E14.5, E15.5, E16.5, E17.5, or E18.5) and for a total 12 different stages by treatment combinations.

From E13.5-16.5 mice were either gavaged with a corn oil control or 125 mg/kg of vinclozolin (n=3-5) solubilized in corn oil. Preliminary experiments showed that in mice 125 mg/kg vinclozolin induces males pups to develop hypospadias 100% of the time and with high hypospadias severity (*125*). Mice assigned to developmental stages E13.5-16.5 were euthanized with isoflurane 1 hour after being gavaged that day, and those assigned to developmental stages E17.5 and E18.5 were euthanized with isoflurane between 11:00-13:00. Pups were removed from the uterus and weighed. Fetuses were staged based on the Theiler staging system (*126*). If the fetus was greater or less than one of the predicted Theiler stage they were considered a different stage and where removed from analysis as their dosing regimen would have begun one day early or late. Sex of fetuses collected on E13.5 and E14.5 was determined by the presence of sex cords in the gonads. Fetuses lacking of sex cords was recorded as female. From

E15.5-18.5 males have descended testis, while female ovaries remained high in the abdomen near the kidneys. External genitalia were dissected from the body and stored in 10% neutral buffered formalin (Richard Allen Scientific, 5700TS, Kalamazoo, MI) until they were imaged.

External genitalia imaging

The ventral aspect of the external genitalia was imaged on Leica SAP08 at 5x magnification after dissection. The same 47 petri dish with black wax (Xichen, China) was used for every sample. The samples were positioned to capture the ventral aspect of the external genitalia; .25 mm pins (Bioquip, 1208B000, Rancho Dominguez, CA) were used to hold the sample in place for imaging. Leica application suite v.4.9 was used to image samples. 8-10 step focused images were acquired for each sample and Helicon focus v.6.7.1 was used to z-stack images into one complied.

Landmark Digitization

After the samples were imaged they were randomized, renamed, and digitized. Randomization was completed by numerically ordering the dam-pup identifiers in Excel then generating 109 random numbers using R statistical environment "sample ()" function. Random numbers were pasted next to the dam-pup columns and then numerically reordered by the random generated numbers. All of the images were copied and renamed according to the random number assignment. This allowed the researcher to be blind to (exact) stage, treatment, and sex of samples. For digitization, each image was then opened in tpsDIG and 12 homologous landmarks were placed according to predetermined morphological anchors (Table 1). Due to the large amount of morphological change that happens through development and the curved nature of the external genitalia we also used a semi-landmark approach to effectively capture the true shape. Semi-landmarks are not placed on homologous structures as landmarks are, but instead allow for analysis of curved shape change () to characterize stage specific shape. Placing semi-landmarks along curves of the external genitalia allows for information on preputial swelling and glans outgrowth that change significantly through development. Therefore, the semi-landmarks allow for sampling across developmental time where homologous landmarks are difficult to find. Semi-landmark curves were added

to connect all of the anchors and 4-8 semi-landmarks were placed equidistant along the curves (Figure 1A). A total of 64 semi-landmarks were placed on each sample. Once all landmarks and semi-landmarks were digitized the TPS filed output from tpsDIG was opened in tpsUTIL and the curves were appended as actual landmarks. Then the new output file from tpsUTIL was opened in tpsUTIL and the overlap between the semi-landmark points and landmark anchor points were deleted.

Analysis of Shape

TPS output from tpsUTIL was then opened in tpsrelw32 and consensus of all the samples was made and samples were superimposed with Procrustes fit. Partial warps and relative warps were then calculated. Centroid size and relative warp values were then saved in an Excel file. Centroid size is defined as the square root of the sum of squared distances from each landmark to the centroid of the landmark (*127*). Centroid size is used as a continuous measure of size or area of the sample that can be used as a covariate to correct for allometric relationships (*127*). Relative warps are the variation of landmark displacement described and each relative warp can be viewed as a descriptor of shape (*127*). Samples were unrandomized and identifiers of stage, sex, and treatment were included in the data sheet. The first ten relative warp scores described >95% of the variation in shape and were used for all further analyses (Figure 1B).

Statistical Analysis

R statistical environment v.3.4.4 was used to analyze all shape data. The “hclust” algorithm was used to cluster samples based on relative warp 1. Samples were indicated as correctly clustered if the predicted cluster group matched the actual clustering group. MANOVAs were used to test for differences between groups with $p < 0.05$ designated as significant. The “candisc” package was used to do a canonical discriminant analyses on the three-way interaction terms from the MANOVA output. The canonical variate scores from the discriminate analysis were used to investigate effect size differences between groups of the three-way interaction independent of centroid size and other variables.

Results

Major Axis of shape variation

Relative warp 1 described 60% of the total variation in the data set and had a strong linear relationship with the stage of external genital development. In fact, 90% of the variation in genital shape is explained by genital stage (Figure 2B, Supplemental Movie 1, R^2=0.8947, $p < 0.05$). Interestingly, relative warp 1 clustered into three separate subgroups, which consisted of either E13.4-14.5, E15.5-16.5, and E17.5-18.5 (Figure 2B and 2C). The three separate subgroups correctly clustered into the expected groups 96% of the time. The major change in development occurred in preputial swelling expansion and a covering of the glans. Also, there was a loss of the PUO on the ventro-proximal aspect of the genitals.

Allometric relationships change through time

We assayed how centroid size changed through time. Centroid size non-monotonically changed through time (Figure 3A). From E13.5-15.5 there was a 22.3% increase in centroid size, which indicated that a lot of growth occurred during this phase of development. From E15.5-18.5 there was an 11% decrease in centroid size suggesting that size decreased during this time. There was no effect of treatment or sex on centroid sizes across developmental stages.

To test if external genital shape changed as centroid size varied, a MANOVA with the first 10 warp scores (composite shape) as the response and centroid size by stage interaction as the predictor was used. There was a significant centroid size by stage interaction (df=5, F statistic= 2.3191, $p = 3.33x10^{-6}$), meaning centroid size had a different relationship with genital shape (allometry) across stages (Figure 3B). There was no effect of treatment and sex on the allometric relationship ($df_{treatment}$=1, F $statistic_{treatment}$=1.1918, $p_{treatment}$ = 0.3066, df_{sex}=1, F $statistic_{sex}$=1.3257p_{sex} = 0.22845). Canonical discriminate analysis on the interaction term of the MANOVA was used to describe the interaction term independent of other model terms. E13.5 and E14.5 shape had a negative relationship with centroid size, E15.5 and E16.5 had minimal relationship with centroid size, and E17.5 and E18.5 had strong positive relationships with centroid size (Figure 3B). Shape and size scaled differently across developmental stage, but the scaling differed among the three distinct groups where E13.5 and E14.5 were not different from

one another as was the case for E15.5 and E16.5, and E17.5 and E18.5. The three clusters were all analyzed separately in further analyses to allow for proper allometric covariate inclusion.

Vinclozolin exposure disrupts shape sexual dimorphism

MANOVAs were used on each of the three developmental groups to determine sex and treatment differences. Although data was analyzed by group separately, stage was still included in the models to allow for finer temporal resolution (between the two days). Also, and as mentioned above, centroid size was included as a covariate to correct for allometry. For E13.5 and E14.5 there were no differences in sex, treatment, or stage (Table 2), but there was a significant relationship with centroid size and shape (df=1, F statistic= 8.8913, $p=8.104x10^{-5}$). In the second group, E15.5 and E16.5, there were significant effects of centroid size (df=1, F statistic= 4.3619, p=0.005344), sex (df=1, F statistic= 3.9125, p=0.008827), stage (df=1, F statistic= 12.5060, $p=1.446x10^{-5}$), and sex by treatment by stage interaction (df=1, F statistic= 2.9189, p=0.030073) (Table 2). For the third group, E17.5 and E18.5, there was an effect of centroid size (df=1, F statistic= 9.5584, $p=8.736x10^{-6}$), sex (df=1, F statistic= 7.4173, $p=6.145x10^{-5}$), stage (df=1, F statistic= 5.3921, p=0.0005728), sex by stage interaction (df=1, F statistic= 2.9031, p=0.0190055), and an interaction between sex, treatment, and stage (df=1, F statistic= 3.0905, p=0.0140885) (Table 2).

Canonical discriminate analyses showed that for E13.5 and E14.5 there are non-significant differences between sexes and treatment groups due to the great deal of variation between the stage, sex, and treatment groups (Figure 4). Relative warps 3, 8, and 10 had influenced shape differences at these stages. E15.5-16.5 showed sex differences while vinclozolin treatment diminished those sex differences and caused male genitalia to resemble female genitalia and female genitalia to resemble male genitalia, thus reducing sexual dimorphism in shape (Figure 4). For the stages E15.5-16.5, relative warps 1, 4, 5, and 6 had described the most shape differences between external genitalia groups. On E17.5 the difference between males and females became larger and vinclozolin treatment caused male and female genitalia to develop an intermediate morphology between the male and female values (Figure 4). At

E18.5 the natural sexual dimorphism between corn oil control males and females are the largest observed in the data set and again vinclozolin exposure reduced this normal sexual dimorphism (Figure 4). Relative warps 5 and 10 had strongest relative influence on the shape differences between groups for E17.5-18.5.

Discussion

It is well established that during embryonic development the external genitalia becomes sexually dimorphic largely due to the presence of circulating androgens in males and the lack thereof in females (*20, 29, 30*). No studies have quantitatively analyzed the morphogenic changes of the external genitalia as they occur through time and thus it is not known when the external genitalia become sexually dimorphic, where genital structures diverge, and how these changes are affected by anti-androgenic EDCs. Here we showed genital shape allometrecally scales with its size and that this relationship depends on developmental stage. The scaling relationship was most similar among E13.5-14.5, E15.5-16.5, and E17.5-18.5 (Figure 2C, Figure 2D, Figure 3B). This suggests that the allometric relationship between shape and size changes through time in a punctuated manner and that there may be a lag in time between the molecular determinates of dimorphism and the morphogenetic processes that lead to shape alteration. E13.5-15.5 were the only stages that showed an increase in centroid size relative to the next stage (Figure 3A). This suggests that E13.5-15.5 is a general growth phase of development while E15.5-18.5 is a remodeling phase of development. During the growth phase large amounts of cell proliferation and growth may be promoting genital development while cell migration and apoptosis could be involved in the remodeling stage of development. Future studies should investigate the cellular growth dynamics throughout the developing genitals to better understand the underlying nature of the growth relative to shape changes.

As early as E15.5 morphological sexual dimorphism can be observed and both males and females have already responded to vinclozolin (Table 2) suggesting that they are responsive to vinclozolin prior to this stage. This finding is supported by the E13.5 exposure to an anti-androgen, vinclozolin, reducing androgen receptor concentrations on E14.5 (*128*). It is well known that the external genitalia become

sexually dimorphic during embryonic development. Suzuki et al 2002 (*116*) visually identified that the external genitalia of males and females become qualitatively different on E16.5. The characterized differences of males and females on E16.5 was a larger preputial swelling size in males (*116*). Several other studies confirm that the external genitalia are strongly sexually dimorphic by E18.5 (*13, 48, 116, 129*). Females exhibit less lateral and medial size of the preputial swellings with the urethra exiting on the ventral aspect of the genitalia (*116*). In our study we found that the external genitalia became significantly sexually dimorphic as early as E15.5, which then persists and becomes more exaggerated through time to E18.5. our results argue that studies aiming to study sexual dimorphism be directed toward developmental time earlier than E15.5.. Some studies have shown that molecular dimorphisms can be observed as early as E14.5 (*26, 49, 87*). Mafb and Fgfr2iiiB are known to be sexually dimorphically expressed around the proximal urethral opening and the top of the preputial swellings, respectively (*49, 50*). Based on Suzuki's observation our shape difference data strongly correlate with the expression localization of MafB and Fgfr2iiiB. Future studies should directly correlate gene expression with shape change.

Vinclozolin exposure diminishes sexual dimorphism

Vinclozolin is a well-known anti-androgenic EDC that demasculinizes the external genitalia in male mice (*130, 131*). Other anti-androgenic chemicals, flutamide (*132, 133*), procyomide (*114*), and finestride (*26, 47*), also induce similar genital demasculinization in male mice exposed during the genital masculinization developmental period. A previous study has shown that anti-androgens do impact female external genitalia development (*119*). Similar to previous studies, we found that vinclozolin defeminized the female external genitalia, which resulted in more male like external genitalia. Female external genitalia shape alterations are likely contributed to a difference in hormone signaling. It is unclear however, if antiandrogen exposure has direct effects on female genitalia development or if blocking androgen signaling alters steroid synthesis leading to indirect effects through altered estrogen or progesterone signaling. Regardless, vinclozolin is disrupting normal female development in ways not current understood.

Utility for external genitalia shape analysis

Several fruitful discoveries in developmental biology have resulted from analysis of genetic and environmental perturbations to embryogenesis using discrete quantitative measurements (0, 1, 2, etc.) (*134-138*). Limb developmental studies have led to the understanding of several basic biological tenets through quantitative analysis of malformation (*134-136*). Quantitative analysis of phenotype is a valuable asset for understanding the hormonal and genetic drivers of development. With geometric morphometrics we show when and where changes are occurring in the genitalia and how an anti-androgen can disrupt those changes. Although, not commonly used to evaluate genitalia development, studies on craniofacial development have begun to exploit the use of geometric morphometrics to better understand the genetic and environment impact on face development (*98, 139-141*). Enhancer element differences have been found to drive variations in facial shape morphogenesis (*142*). Young et al (*143*) have found that concentrations of sonic hedgehog induced differences in rostral outgrowth and lateral distance between the eyes and width of the nose. The differences in face shape development were largely attributable to difference of cell proliferation and apoptosis in the regions of developmental change (*143*). Future studies of geometric morphometrics on the external genitalia should focus on the mechanisms that drive sexual dimorphism and the impact of various EDCs on shape change. With this strategy we can begin to evaluate the differences in effects induced by anti-androgenic chemicals and estrogenic chemicals. This will allow for a better understanding of how androgen and estrogen signaling ratios may counteract each other during development, or if they function to control different aspects of development. In addition to understanding how alternate EDCs impact genitalia development, geometric morphometrics can serve as a phenotypic anchor for molecular approaches so we can begin to link molecular mechanisms and morphogenic processes.

Acknowledgements

We would like to thank Ariel Fricke, Josh Mogus, and Sahiti Marella for help with dissections. Thanks to Ed Stellwag, Elizabeth Ables, and Jacques Robidoux for helpful edits and comments.

Figures

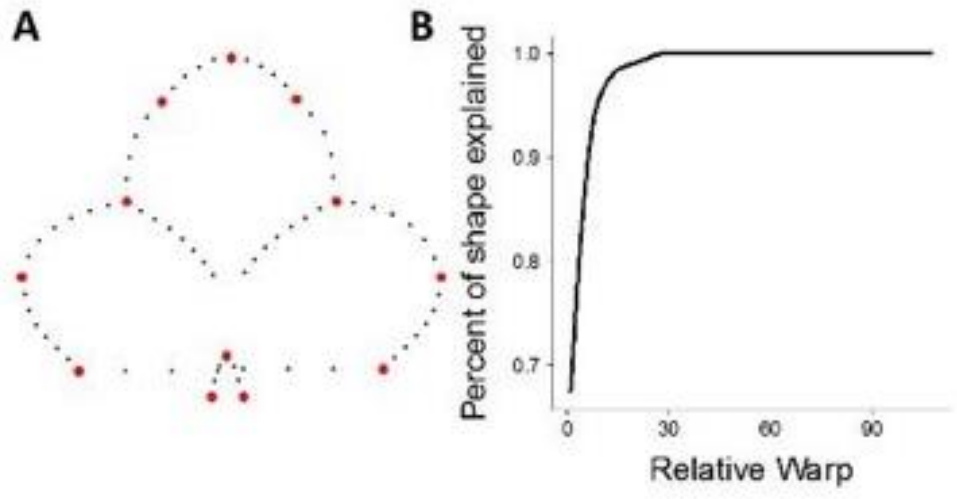


Figure 2. 1 Variation explained by relative warps.

A) Concensus shape of the landmarks (red) and semilandmarks (black). **B)** The first ten relative warps explain >95% of variation in the data. The first relative warp alone explains 60% of the shape variation

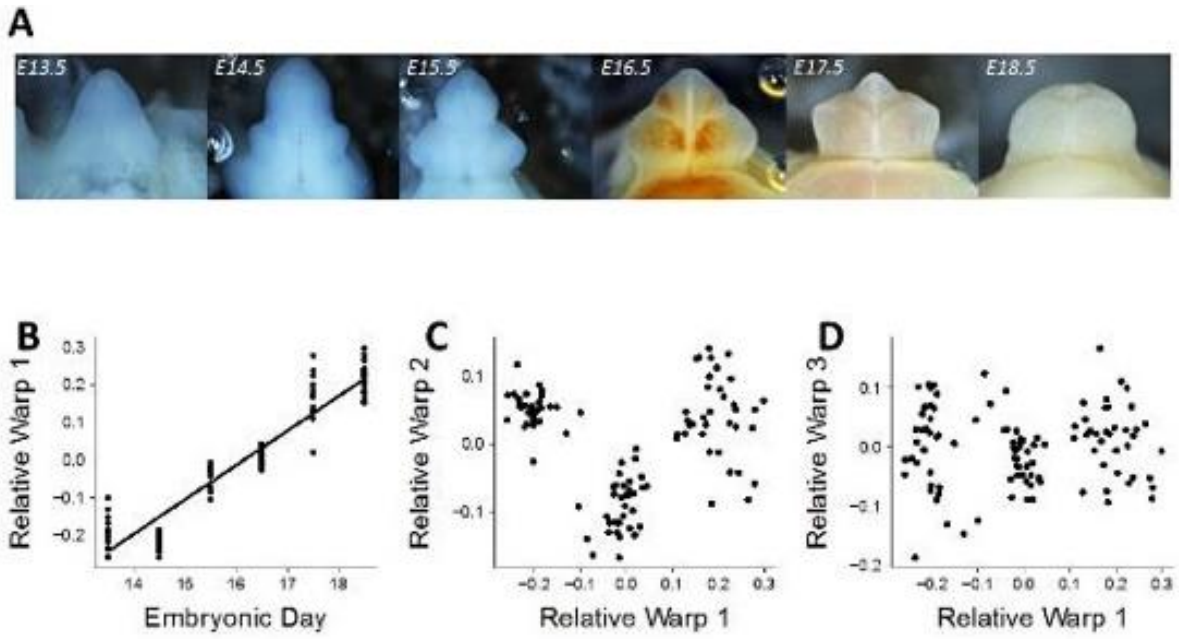


Figure 2. 2 Majority of shape change occurs across developmental stage.

A) Representative specimen images of each developmental stage analyzed. **B)** Large amounts of shape change can be observed in the mouse external genitalia from E13.5-18.5. **C)** Relative warp 1 strongly correlates with developmental stage (R^2=.8947). Relative warp 1 displays shape variation in the preputial swelling height, preputial swelling width, and glans size (Supplemental Movie 1). **C and D)** Relative warp 1 clusters in three distinct groups which include 1) E13.5-14.5, 2) E15.5-16.5, and 3) E17.5-18.5.

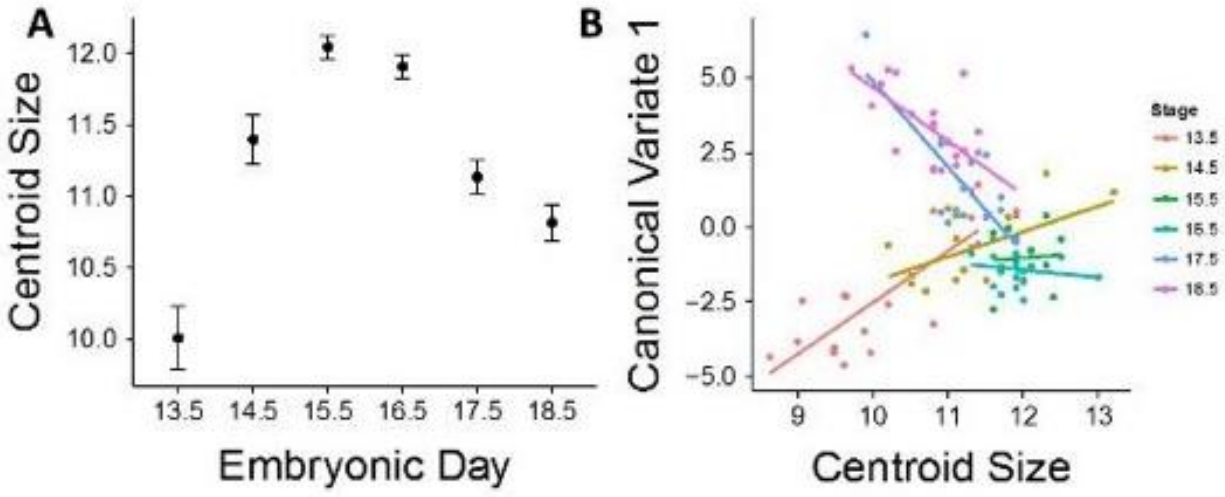


Figure 2. 3 Shape differentially scales with stage-dependent change in centroid size.
A) Centroid size showed a large increase from E13.5 to E14.5 to E15.5. Starting at E15.5 centroid size begins to decrease through E18.5. **B)** Depending on stage centroid size has a different relationship with external genitalia shape. E13.5-14.5 show a positive relationship, E15.5-16.5 show no relationship, and E17.5-18.5 show a negative relationship to centroid size.

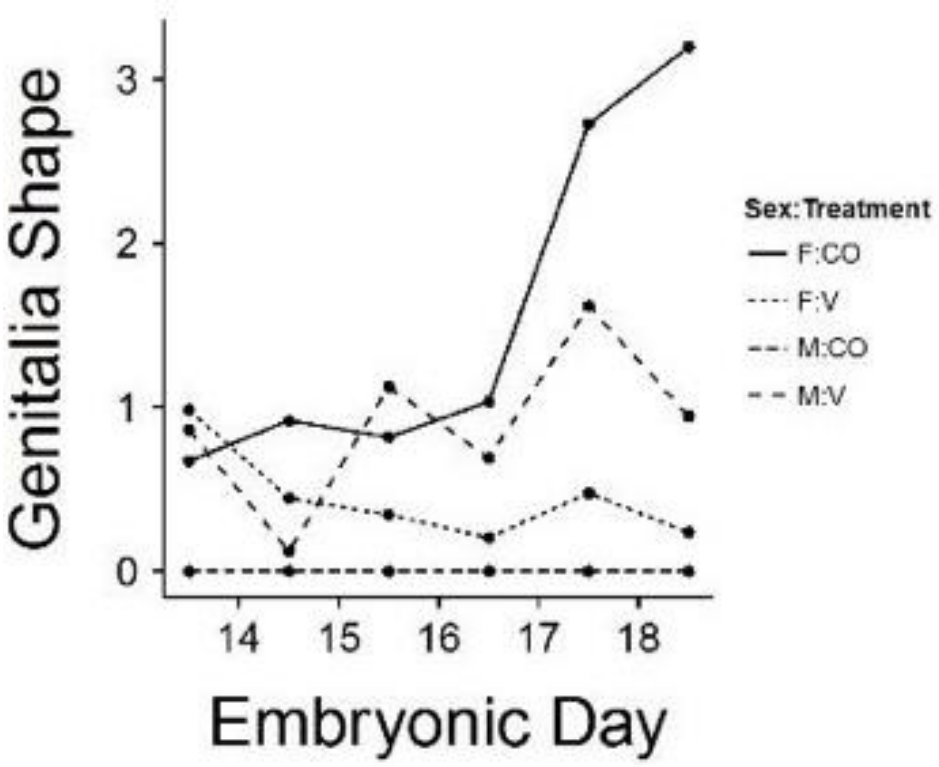


Figure 2. 4 Vinclozolin exposure reduces sexual dimorphism in external genitalia
. Genitalia shape indicates canonical variate 1 from the three-way interaction between sex, treatment, and stage in the MANOVA analysis. E13.5-14.5 show that there may be differences in morphology at this early stage of development, although non-significant. On E15.5 sex and treatment differences can begin to be observed. Vinclozolin exposure reduced the observed sexual dimorphism. Sex and treatment differences persist through E18.5.

Landmark number	Landmark description
1	Distal tip of the external genitalia
2	Distal most indentation on the left of the glans
3	Position on the left where the glans meets the preputial swelling
4	The leftmost lateral point of the preputial swellings
5	Position where the leftmost aspect of the genitalia connects to the perineum
6	Leftmost opening of the proximal urethral opening
7	The top of the proximal urethral opening
8	The right most opening of the proximal urethral opening
9	Position where the right aspect of the genitalia connects to the perineum
10	The right widest point of the preputial swellings
11	Position on the right where the glans meets the preputial swelling
12	Distal most indentation on the right of the glans

Tables

Table 2. 1 Landmark order and description

Table 2. 2 P-values from MANOVA analysis

Stage Comparisons	Centroid Size	Sex	Treatment	Stage	Sex: Treatment	Sex: Stage	Treatment: Stage	Sex: Treatment: Stage
E13.5-14.5	**0.00007**	0.13385	0.23233	0.03838	0.21033	0.49683	0.69175	0.94452
E15.5-16.5	**0.038281**	**0.007056**	0.604738	**0.00001**	0.415839	0.460035	0.681324	**0.015420**
E17.5-18.5	**0.000005**	**0.001408**	0.308435	**0.003550**	0.960449	**0.018920**	0.076759	**0.007027**

Chapter 3: A validated protocol to quantify severity of male urogenital feminization using the MOUSE (Mouse Objective Urethral Evaluation)

Abstract

Background

Congenital abnormalities vary in presentation, yet studies using model organisms tend to focus on occurrence rather than severity of the defect. Scoring severity of abnormalities in model systems allows explicit hypothesis testing during basic, translational, and reverse translational studies. We developed and validated a protocol to quantify severity of male urogenital feminization (hypospadias) in the mouse model. Hypospadias is one of the most common birth defects in the world.

Methods

To induce genital feminization, pregnant mice were exposed to different concentrations of the anti-androgen vinclozolin. Genitalia were photographed at gestational age 18.5. A dichotomous scoring system to evaluate genital feminization was developed, and validated against histological measurements of urethral length. A training protocol was developed for novice scorers, and criteria were defined to evaluate precision and accuracy of scores.

Results

Vinclozolin induced variation in hypospadias severity. Severity scores were tightly correlated with histologically determined urethral length and both techniques showed similar dose response relationships. Novice observers were trained to precisely and accurately score hypospadias severity.

Conclusion

This standardized scoring system advances the mouse as a model to study urogenital development, and will facilitate research on the mechanisms driving genital feminization in males, and aid translational hypospadias research.

Introduction

Morphological variation occurs because developmental and molecular mechanisms driving morphogenesis differ across genotypes and environments (*144*). Many traits, however, are studied in a binary context: present or absent; normal or abnormal. This framework limits our ability to build a complete, and integrated understanding of the mechanisms responsible for morphogenesis because developmental processes do not induce binary outcomes. The scoring system we develop and validate provides a fine scale quantification of the severity of a common genital birth defect so that the mechanisms underlying differences in genital morphogenesis can be studied.

Congenital abnormalities induced by altered genital morphogenesis are surprisingly common in humans. For example, hypospadias is the second most common birth defect in the U.S.A., occurring in almost 1% of males. In other regions of the world, rates are as high as 4.6% (*145*). Hypospadias occurs when the urethra is shortened and opens along the shaft of the penis, rather than at the distal tip. It has a complex etiology and broad variation in its presentation (*146*). Hypospadias severity in newborns varies from requiring no intervention, to necessitating complete sex reassignment surgery and vaginoplasty (*147*).

In fact, the Hypospadias Objective Penile Evaluation (H.O.P.E.) scoring system is used, in humans, to evaluate the severity of several surgically correctable genitalia abnormalities (*148*) and facilitates evaluation of both preoperative options and postoperative outcomes. Determining

the molecular and cellular mechanisms underlying the variation in genitalia abnormalities will advance our understanding why they are occurring in humans, and aid development of therapies to reduce their occurrence and incidence. Such mechanistic studies are most efficiently conducted in established model systems that exhibit similar development and responsiveness to environmental perturbations. Mice are the accepted model for investigating urogenital development (*149*). As in humans, hypospadias severity varies in mice. However, current techniques to evaluate severity of genital feminization and hypospadias in the mouse require destructive techniques such as histology (*150*) or resin casting (*151*), which allow measurement of urethral length (which is shorter with hypospadias), but limit the use of tissues for genetic, physiological, or developmental studies.

Reproductive abnormalities in the rat increase in severity with mixtures of endocrine disrupting chemicals (EDC) (*152*), and many studies suggest that the increase in incidence of hypospadias in humans is related to fetal EDC exposure (*153*). Here we develop the Mouse Objective Urethral Severity Evaluation (M.O.U.S.E), as a standardized scoring system for the mouse that allows efficient and consistent comparisons of hypospadias severity to facilitate mechanistic studies, and comparisons across genetic and environmental manipulations. This scoring system is validated against urethral histology, the accepted endpoint used to evaluate hypospadias severity, (*154*). We go further to show that scores are negatively associated with anogenital distance (AGD), a well accepted marker of masculinization. We provide a training protocol that includes guidelines for determining when observers can precisely and accurately score severity and show that scorers with different educational backgrounds can properly use the training protocol. M.O.U.S.E. provides a single measurement through which multiple

laboratories can exchange information about how genetic manipulations or environmental treatments influence genital development.

Results

To develop M.O.U.S.E. as a standardized hypospadias severity scoring protocol, we needed to compare individuals with a range of hypospadias severities. To induce hypospadias at different severities, pregnant mouse dams (N=3) were exposed to one of four doses of the androgen antagonist vinclozolin (0, 100, 125, 150 mg/kg) every day for four days during the critical period for genitalia development (gestational age (GA) 13.5-16.5) (*155*). Genitalia of each pup (GA18.5) was photographed in a standard position (see Supplemental Methods (online)), histologically processed, and evaluated based on predetermined landmarks (Figure 1, panel a). Genital length scaled with urethral length at the same rate across all individuals (χ^2=40.598, p= 1.45e-07, slope (B_1)= 0.3205). Across all treatments, males displayed 33% larger urethral lengths (χ^2=108.47, p= 2.2e-16) than control (0 mg/kg vinclozolin) females. However, males from vinclozolin treated groups exhibited a saturating dose-dependent shortening of urethral length (χ^2=18.307, p=1.88e-05, Figure 1, panel b) relative to control males.

Next, we created a dichotomous key with defined scoring criteria that distinguished among: normal (0), abnormal (1), feminized (2), and severely feminized (3) penis structure, based on comparing overall genital morphology and location of the urethral exit (Figure 2 and Supplemental Methods (Online)). We used the dichotomous key to evaluate pictures of genitalia generated in the dose response study. Analogous to urethral length, hypospadias severity using M.O.U.S.E scores showed a saturating dose dependent increase in hypospadias severity between 0-150-mg/kg vinclozolin (χ^2=12.783, p= 2.858e-05, Figure 3).

To validate that M.O.U.S.E. accurately represents hypospadias severity we regressed MOUSE severity scores to histologically determined urethral lengths and found a significant negative relationship; 97% of the variation in urethral length was explained by MOUSE hypospadias score (R^2= 0.9718, slope= -1.5467; Figure 4, panel a). To further confirm that the M.O.U.S.E. scores provide valid information about masculinization, severity scores and anogenital distances (AGD) for male pups from each dam were separately averaged and then regressed against one another. Indeed, mean score was negatively related to mean AGD (R^2=.6189, p= .00146, Figure 4 panel b). In fact, 62% of the variation in AGD could be explained by differences in hypospadias severity.

To determine if multiple people with diverse educational backgrounds could use the M.O.U.S.E scoring system we trained two novice scorers, with different educational experiences, (a biology graduate student and a high school English teacher) to score genitalia. Each person was trained for only 10 minutes prior to scoring. Important landmarks were indicated, and the trainer discussed the process of scoring using practice pictures, which were not included in the examination set (Figure 2 & Supplemental Methods (Online)).

Precision was evaluated in two ways. First, observers scored a single set of 24 sample photographs two times and a Pearson's correlation was run to determine if the two scoring attempts were consistent. A correlation coefficient of r = 0.8 between the two attempts was required to be considered precise. Second, a paired t-test was run on the two scoring attempts to ensure that the difference in scores did not significantly deviate from zero (so that attempts were not different from one another). It is possible for scorers to be consistent but score differently between scoring sessions (e.g., consistently scoring lower in the second session). Quantifying precision in these two distinct ways is rigorous and necessary for standardized results. Accuracy

was evaluated by comparing M.O.U.S.E scores to the histologically determined urethral length. To be considered accurate, urethral length and severity score had to show a significant negative relationship (urethral length decreases as severity increases) and have a $R^2 \geq 0.95$ (see Supplemental Methods (Online) and http://thescholarship.ecu.edu/handle/10342/5650, for more methodological detail, practice pictures and corresponding histological data).

If the observers failed any precision or accuracy test they were retrained on new practice pictures and underwent the scoring process a second time using new sample photographs. Both the graduate student and high school teacher required 2 training sessions and a total of 45 and 30 minutes (respectively) across both scoring sessions to meet the criterion described above (Table 1), at which point both individuals were considered capable of scoring hypospadias severity in future experiments. To facilitate standardized severity scoring across laboratories, we have provided a protocol for taking standardized photographs, a detailed training guide, genitalia photographs, and corresponding histological data in the Supplemental Methods (Online).

Discussion

M.O.U.S.E. is an easy to use, standardized scoring system that allows accurate and precise quantification of hypospadias severity. All scoring is completed on photographs of the genitalia, and thus is non-destructive. The use of photographs to evaluate morphology is powerful for several reasons. First, pictures allow scorers to be blind to experimental treatment, pup sex, and dam. Second, the use of pictures frees the genitalia tissue to be used for mechanistic research. Individual samples can be dissected, photographed, and then processed in a variety of ways rather than having to be preserved for later randomization and scoring. Finally, photographs can be easily stored, shared, distributed, and scored multiple times to obtain average severity scores for each individual. Typical protein and mRNA preservation techniques require the tissues to be

preserved in noxious solutions that change morphological structures and make multiple observations across time difficult.

Another advantage of scoring genitalia morphology using M.O.U.S.E rather than relying on a single measure, such as position of the urethral opening (e.g., 2/3 of the way down the shaft), is that many aspects of morphology are integrated into a single M.O.U.S.E. score. Hypospadias is a disorder that affects more than urethral length. M.O.U.S.E. assesses: the extent of outgrowth of the preputial swellings, overall penis shape (relative to normal males and females), how tightly the preputial swellings surround the glans, glans shape, as well as position and size of the urethral meatus. The increased resolution that M.O.U.S.E provides for evaluating genitalia morphology will enhance basic and applied research.

Advancing the mouse model of hypospadias

The M.O.U.S.E. scoring system provides a sensitive method to determine if penis morphology is affected even when incidence of hypospadias remains consistent across treatments. The increased sensitivity of M.O.U.S.E will facilitate comparisons of hypospadias severity across studies using different genetic mutants and will help build a synthetic understanding of penile organogenesis and urethral tube closure. Developing a more synthetic knowledge of the molecular drivers of penile development in the mouse will facilitate our understanding of the mechanisms through which hypospadias occurs in humans.

This type of standardized scoring system is also essential to investigate the multifactorial nature of hypospadias. Evaluating the complex physiological and developmental processes involved in driving hypospadias severity is made possible when more discrete measurements are recorded and used to test explicit mechanistic hypotheses. M.O.U.S.E. scores can be quantitatively compared with measures of androgen levels, gonadal function, AGD (Figure 4,

panel b), and other morphological or behavioral outcomes. With these types of data, we can integrate multiple morphological and physiological changes into a synthetic understanding of genital development and function.

M.O.U.S.E., will facilitate our understanding of the genetic and environmental drivers of hypospadias severity and advance translation of basic urogenital research. For example, human polymorphisms in several genes are significant risk factors for development of hypospadias (*156-158*). With M.O.U.S.E. we can conduct reverse translational work to determine which human gene mutations or combinations of mutations lead to more or less severe phenotypes in rodents, so that we can further study causal mechanisms. Furthermore, with M.O.U.S.E., researchers can test putative environmental factors associated with hypospadias in humans, and determine which induce the most severe hypospadias in the mouse. This approach will provide insight into what chemicals pregnant mothers should avoid. M.O.U.S.E. will also aid in development of therapies to reduce hypospadias severity. For example, previous epidemiological studies have shown that exposure to multivitamins generally (*159*) and folate specifically (*160, 161*) reduces the risk hypospadias in newborns. M.O.U.S.E. will facilitate research identifying which nutritive supplements reduce the severity of hypospadias most dramatically.

The M.O.U.S.E. scoring system strengthens the mouse as model for evaluating the mechanisms driving congenital defects, and provides a means through which to evaluate sensitivity to specific chemicals and responses to therapeutic agents. Our work also advances the mouse as a model to study basic developmental and physiological processes that drive urogenital development.

Methods

Mouse maintenance and treatment

All studies were approved by the East Carolina University Institutional Animal Care and Use Committee (AUP D-297). Eight-week-old CD-1 mice (Charles River Laboratories Raleigh, NC) and acclimated to 70-72 °F on a 12 h light-dark cycle with free access to food and water (Purina ISOCHOW St. Hager City, WI) for at least seven days prior to experimentation.

Generating variation in hypospadias severity

To generate differences in hypospadias severity in the mouse that phenocopies the variation seen in humans with hypospadias, we conducted a dose response experiment fully replicated three times using vinclozolin (Sigma Aldrich, *St. Louis, MO*), a model anti-androgenic endocrine disrupting pesticide. Vinclozolin and its metabolites are known to competitively inhibit testosterone from binding to the androgen receptor and have been used to consistently induce hypospadias in previous studies (*45*, *151*, *162*). Acclimated nulliparous females were placed with male mice and checked every morning thereafter for vaginal plugs. Presence of a vaginal plug signified copulation occurred the previous night and that morning was considered gestational age (GA) 0.5. Pregnant dams (N=3/dose) were dosed on GA 13.5-16.5 with 0, 100, 125 or 150 mg/kg of vinclozloin. Tocopherol stripped corn oil (Millipore, *Billerica, MA*) was used as a vehicle and for vehicle control (0mg/kg dose). During dosing, females were housed with same treatment dams or singly. To remove variation in developmental stage (age) caused by variation in day of birth (e.g., if parturition onset is affected by the treatments), females were humanly sacrificed on GA 18.5 and fetuses were removed. Sex was determined by examining the gonads (gonad morphology is not affected at the exposure time window-data not shown), and developmental stage of the embryo was verified by morphological evaluation (Theiler stage 26) (*126*). AGD was measured with a micrometer fitted on a Leica M80 stereoscope (Buffalo Grove,

IL). Genitalia were removed, and photographed at 4x zoom with a Leica M165FC stereoscope (Buffalo Grove, IL) using the z-stack add-on for Leica Application Suite software. Samples were then stored in 10% neutral buffered formalin (Fisher Scientific, Waltham, MA) until later histological processing. At GA 18.5 the genitalia are clearly sexually dimorphic, and hypospadias (shortened urethra which opens more proximally than normal) is observable both visually and histologically. To minimize the probability of misclassification of hypospadias severity, samples need to be photographed in a standard position, pictures should be clear, especially where the urethra exits, and focus should be on the ventral aspect of the penis (for a full description see the Supplemental Methods (Online)).

Scoring hypospadias severity

When scoring, one observer, blind to treatment, scored all males in one attempt. All scoring was completed on photographs and not live or newly euthanized individuals. In practice, hypospadias severity is continuous and scores can technically lie between 1 and 2 or 2 and 3. In these situations observers can use smaller score intervals (e.g., 1.5 or 2.5). Or, observers can provide the score that is best suited (e.g., closest integer score), and samples can be scored multiple times to obtain an average score per individual across attempts, which will more accurately estimate the true hypospadias score (we used this latter approach).

Validating scores

We validated the gross morphological scores of hypospadias severity by comparing them to histologically determined urethral lengths and AGD (an accepted biomarker of urogenital masculinization). Genitalia were dehydrated, infiltrated, and embedded vertically in paraffin wax, cross-sectioned at 10 μm, and stained with hematoxylin and eosin. Each sample was sectioned entirely and data collected included the number of the section that contained the base of the penis, the urethral exit, and the tip of the penis (Figure 1, panels a & b). The urethral

length and total penis length were measured by counting sections from the base of the penis to exit of the urethra and the tip of the penis, respectively, then the total number of sections for each endpoint was multiplied by section thickness (10 µm). The base of the penis was defined as the first section not attached to perineal epidermis (Figure 1, panel a). Urethral exit was the first section where the urethral epithelium opened to the environment, and adjacent epithelial walls were not touching (Figure 1, panel a). The tip of the penis was identified as the last observable tissue on the slide.

Individuals are different sizes and thus have variable penis sizes, and the length of the urethra is dependent on penis size. To account for this correlation, urethral length was corrected for penis size. To obtain the relationship between urethral length and penis size we used a generalized linear random effects model, with dam treated as a random effect. Using urethra to penis length ratio is not an appropriate correction method due to the non-isometric relationship between penis size and urethral length at this developmental stage. To account for the allometry between urethral length (y) and penis size (x), we used a power law correction according to Equation 1 (*124*).

$$y / x^{a} = k \qquad \text{(Equation 1)}$$

We used three approaches to validate our scoring system. First to insure that the scores we defined provided a fine enough scale to accurately classify individuals with different urethral lengths (the common marker for hypospadias) we visually compared the dose response generated from the M.O.U.S.E scores to the dose responses generated from the urethral lengths (µm) determined histologically (Figure 1, panel c and Figure 3). This allowed us to determine if a similar dose response relationship was obtained with each data set. Second, to insure that each of

our defined scores captured discrete non-overlapping categories of urethral lengths, the histologically determined urethral lengths for individuals falling within each score severity category were averaged and regressed against score (Figure 4, panel a). Here, if M.O.U.S.E scores capture true histological differences in urethral length then score and mean urethral length should be highly related (Figure 4, panel a). Third, we compared the M.O.U.S.E scores and AGDs to ensure that the hypospadias severity scores we generated were negatively associated with a well-accepted biomarker of masculinization. To test for a significant association between these measures, M.O.U.S.E scores and AGDs of male pups within each dam were averaged and regressed against one another.

Validation of our training protocol

To determine if multiple people with diverse educational backgrounds could use the standardized scoring system, we trained two individuals with different technical qualifications to score hypospadias severity using M.O.U.S.E. One individual was a graduate student in our laboratory who had no experience working on genitalia and the other was a high school English teacher with no formal scientific training. Each trainee was taught to recognize morphological landmarks, and score with a subset of photographs of mouse genitalia from the dose response experiment for which we had histologically validated urethral lengths (see Supplemental Methods (Online) (http://thescholarship.ecu.edu/handle/10342/5650)). To assist researchers in learning how to follow the scoring protocol, we provide the detailed training guide, pictures, and corresponding histological data as Supplemental Methods and Figure S1 (online). Here, we detail the criteria used to evaluate whether a trainee is scoring properly and is ready to begin scoring experimental data.

After being trained, each scorer was provided with one set of 24 test pictures and asked to score each picture two times within the time span of two days. To be considered an adequate scorer, individuals had to show that their scores were both precise and accurate.

We determined precision in two ways. First, a Pearson's correlation between the two scoring attempts (for each scorer separately) was run to determine if the scorer was consistent in the way they scored single samples. A Pearson's correlation coefficient > 0.8 was considered the threshold correlation coefficient. The second measure of precision was a paired t-test where we asked if the difference in scores obtained deviated significantly from zero. This tested whether the scores from the first and second scoring attempts were significantly different from one another and ruled out any potential positive or negative bias that cannot be detected in a Pearson's correlation (scores might be correlated but not the same). When the Pearson's correlation coefficient was > 0.8, and difference in the two scores did not significantly differ from zero the scoring was considered precise. If the individual showed acceptable internal consistency (precision) between scores, then we evaluated accuracy. If scores did not meet these criteria, scorers were retrained and provided with a second (different) set of test pictures.

Accuracy was evaluated by comparing the second (or last data set of) hypospadias scores, which is assumed to be the most accurate, to the histologically determined urethral length (μm). If urethral length significantly negatively correlated with the observer M.O.U.S.E. score (threshold R^2=0.95 for averaged values) the trainee was deemed a trained precise and accurate hypospadias scorer. If the individual failed any of the precision or accuracy checkpoints they were retrained and asked to rescore a new set of 24 pictures, and undergo the process again. The trainee alternated between sets of randomly selected training pictures until they passed all the

checkpoints (photographs, protocols, and histology data are provided in the Supplemental Methods (Online)).

Statistical Analysis

All data were analyzed using R statistical programming environment v. 3.1.2. Statistics used for scoring validation and within the training protocol are detailed above. Generalized linear mixed effects models (GLMM) from the lme4 R package (*163*) were used to analyze male and female urethral lengths as well as the dose effect on urethral length and hypospadias severity score in males. Dam was treated as a random effect. Stepwise likelihood ratio tests were used to evaluate the importance of parameter inclusion into the model. The effects package was used to extract the fitted values and confidence intervals from the GLMM (*164*).

Acknowledgements

We thank J. Kelly and B. Blake for recording detailed notes and participating in the training protocol and A. Helms for providing helpful comments and edits. We would also like to thank M. McCoy for help with project development and data analysis.

Supplemental Methods

Photograph protocol

Materials

- Experimental animals
- Dissection tools
- 47mm Petri Dish
- Black wax (XICHEN® Carved Wax Sealing Sticks, Amazon.com)
- 1X PBS
- Insect pins
- Stereoscope equipped with a camera

Protocol

- Melt black wax and fill petri dish until a quarter full

- Have petri dish filled with solidified black wax and cold 1X PBS ready when dissecting
- Remove and humanely sacrifice pups from dam
- Remove lower portion of pup
    - Cut with scissors just above the ilium and below the ribs
    - Remove the legs and tail of the pup
- Document sex
- Stick pin through the dorsal portion of lower body approximately half-way down the back until it exits ventrally (be mindful not to pierce the genitalia)
- Stick pin into wax
- Genitalia must remain under 1X PBS free of air bubbles
- Position genitalia under the stereoscope until the urethral meatus is clearly visible and the orientation resembles Figure 2 and Supplemental Figure 1
    - Multiple pins may be required to hold down the sides of the lower body
    - Ensure genitalia remains under PBS
- Take picture
- Use multi-image/stack software (e.g. LAS Multifocus, Photoshop, etc.) to increase the quality of the pictures

Training protocol

Preparation

- Print dichotomous key (Figure 2) for reference
- Read through dichotomous key
- Examine practice pictures with descriptions of landmarks and relate back to the dichotomous key

Scoring

- Open one of the provided zip folders (http://thescholarship.ecu.edu/handle/10342/5650) with 24 randomly assorted photos
    - The order of these photos correspond with the order of the provided histology
- Examine one picture at a time and give each penis a score, record the score in a spreadsheet (e.g., excel)
- In preparation for the second scoring attempt re-randomize the set of photos
    - Keep track of which new numbers correspond with the old numbers
- Within a week, but at least 24 hours later) rescore the newly randomized set of photos in a different sheet of the workbook
- Unrandomize the second scoring attempt so that data corresponds to histology data
    - Order the scores by the old identifying numbers
- Compile the two scoring attempts into one spreadsheet
- Copy and paste the histology ratio column provided in the sheet below into the spreadsheet
    - 1.1, 1.2, 1.3 … corresponds to training set one picture 1, 2, and 3 etc.

- 2.1, 2.2, 2.3 … corresponds to training set two picture 1, 2, and 3 etc

Analysis

- Run analyses in statistical program of preference (R, SPSS, etc)
  - Pearson's Correlation on the two scores. (Precision)
    - r>.8 (High correlation)
  - Paired T-test comparing two scoring attempts. (Bias)
    - P-value >.05 (No difference)
  - R^2 of histology ratio averaged across given score from second or last attempt. (Accuracy)
    - R^2>.95 (high explanatory value)
- If all thresholds are met, then scoring is considered both precise and accurate, and genitalia abnormality severity can be scored in experimental animals
- If failed restart process with second scoring set

**** If training attempts exceed two, the two sets can be combined and 24 random pictures can be picked from the 48*

Tables

Table 3. 1Members of the public and graduate students can be quickly trained to score hypospadias severity.

Each trainee required two trials to successfully score hypospadias severity. [a] indicates where trainee violated criteria.

Occupation	Trial	Time to Complete	Precision (Pearson's r)	Precision (p-value)	Accuracy (R^2)
Graduate Student	Trial 1 Trial 2	34 minutes 14 minutes	.700[a] .860	.030[a] .714	.841[a] .991
High School Teacher	Trial 1 Trial 2	18 minutes 12 minutes	.850 .870	.001[a] .714	.929[a] .984

Figures

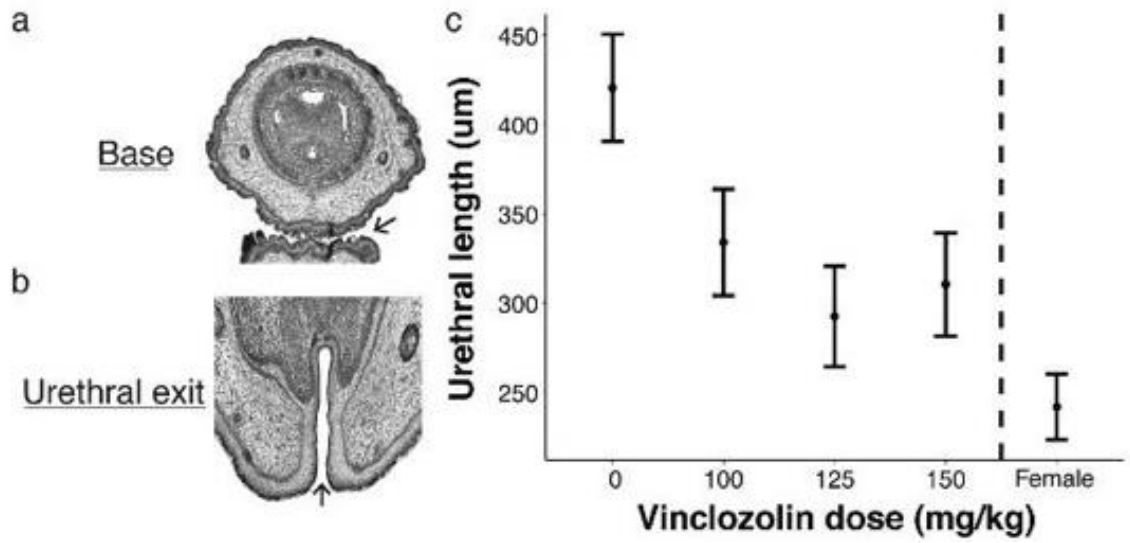


Figure 3. 1Variation in hypospadias severity induced by vinclozolin.

a,b) Histological images displaying the landmarks used for calculating urethral length. a) Arrow indicates the first section where the penis is completely separated from the perineum and was recorded as the first section of the penis. b) Urethral exit was counted as occurring on the first section where the urethral tube opens to the environment (arrow). c) Urethral length exhibited a saturating dose response decreasing from 0-150-mg/kg (p=1.88e-05; N=3 dams). Error bars represent 95% confidence intervals.

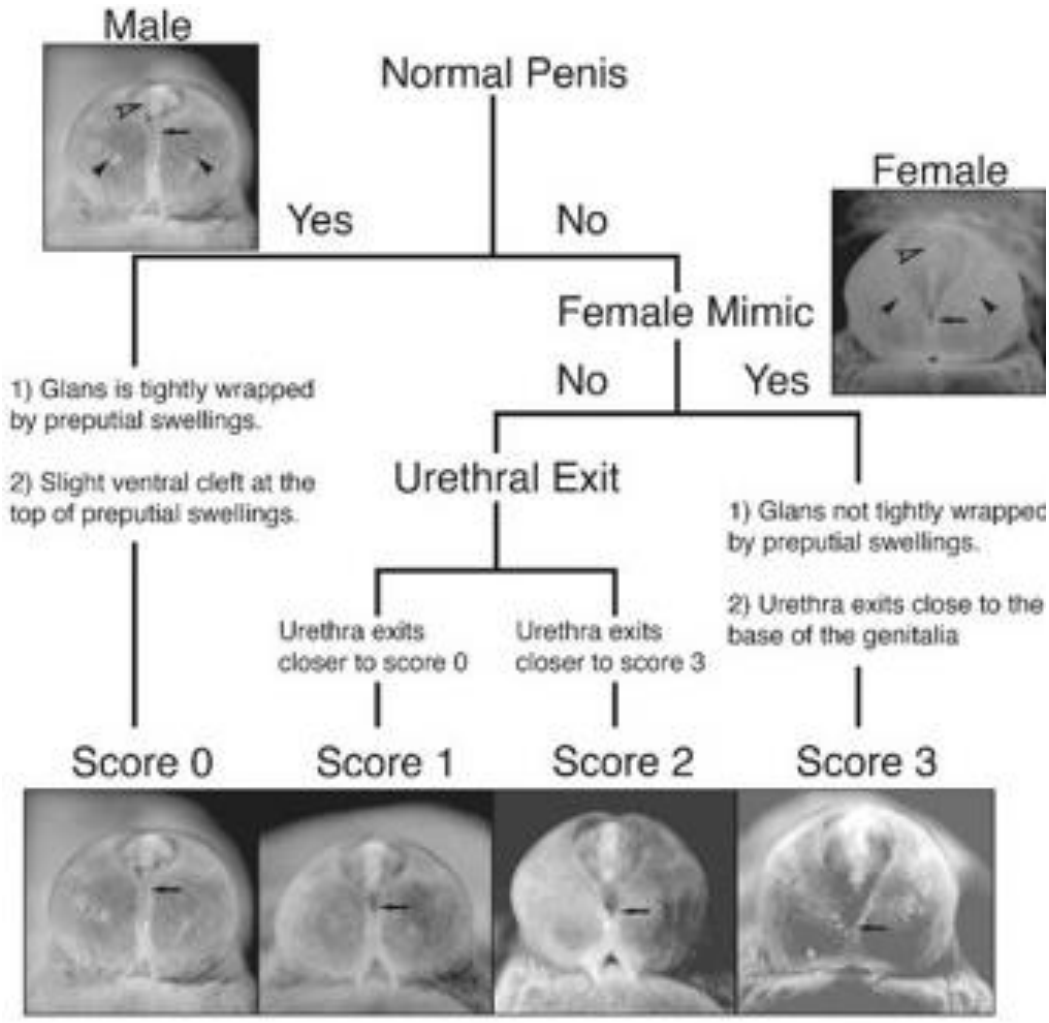


Figure 3. 2Evaluating hypospadias severity.

Mouse objective urethral score evaluation (M.O.U.S.E.) is a dichotomous key that uses a stepwise method for scoring hypospadias severity in the mouse. The observer first asks if the penis is normal, defined as having a circular glans (hollow arrowhead) tightly wrapped by preputial swellings (solid arrowhead) and only a slight ventral cleft at the distal fusion of the preputial swellings (arrow). When the penis is considered normal, it is scored as zero. If the penis is not normal, the observer asks if the morphology mimics a female. In females the clitoral glans is not tightly wrapped by the preputial swellings, much of it is clearly visible, and the

urethra exits below mid shaft (arrow). When the penis phenocopies a female genitalia, it is given a score of three. If the penis is neither normal nor completely feminized then it is scored as one, if the preputial swellings are wrapped around the glans of the penis forming an oval shaped glans but the urethral meatus is more proximal than normal. A score of two is recorded when the glans was not tightly wrapped, and the urethral exit occurs near mid shaft; See Supplemental Methods (Online) for more details.

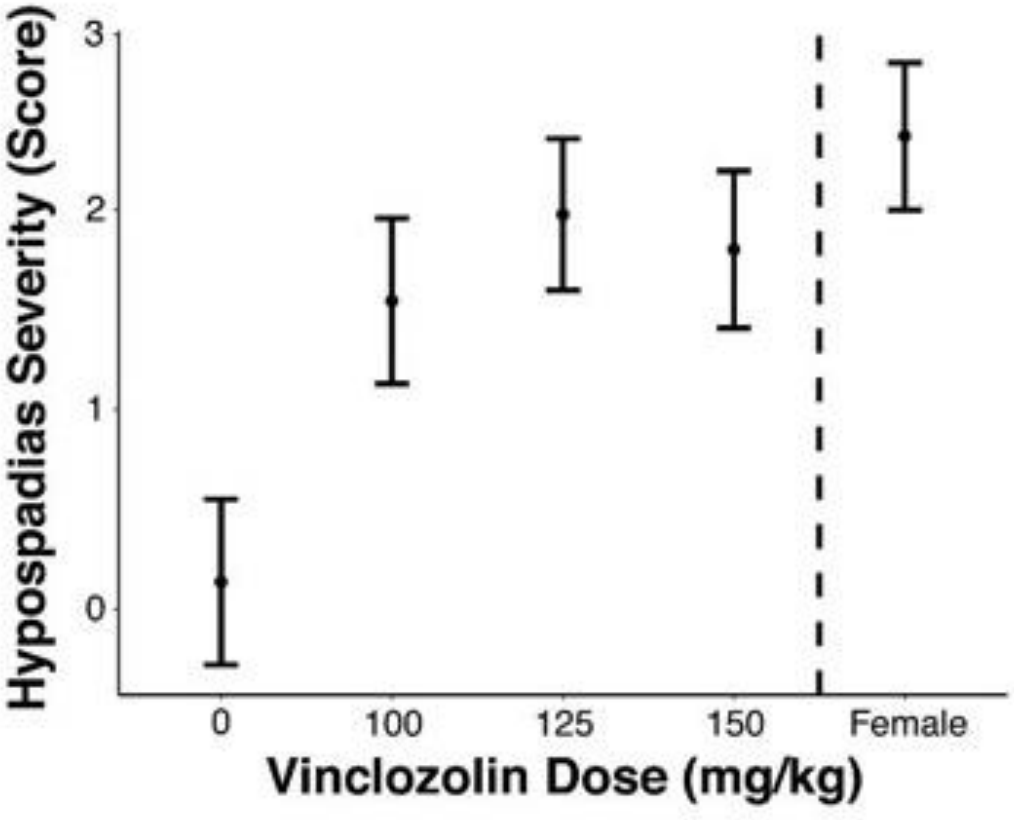


Figure 3. 3 Scoring hypospadias severity.

Hypospadias severity score scales significantly with vinclozolin dose (p= 2.858e-05 ; N=3 dams). Hypospadias severity exhibited a saturating dose response decreasing from 0-150-mg/kg similar to the pattern of urethral length (Figure 1). Females (scored blind) have the highest

"hypospadias severity" when compared to males from each dose. Error bars represent 95% confidence intervals.

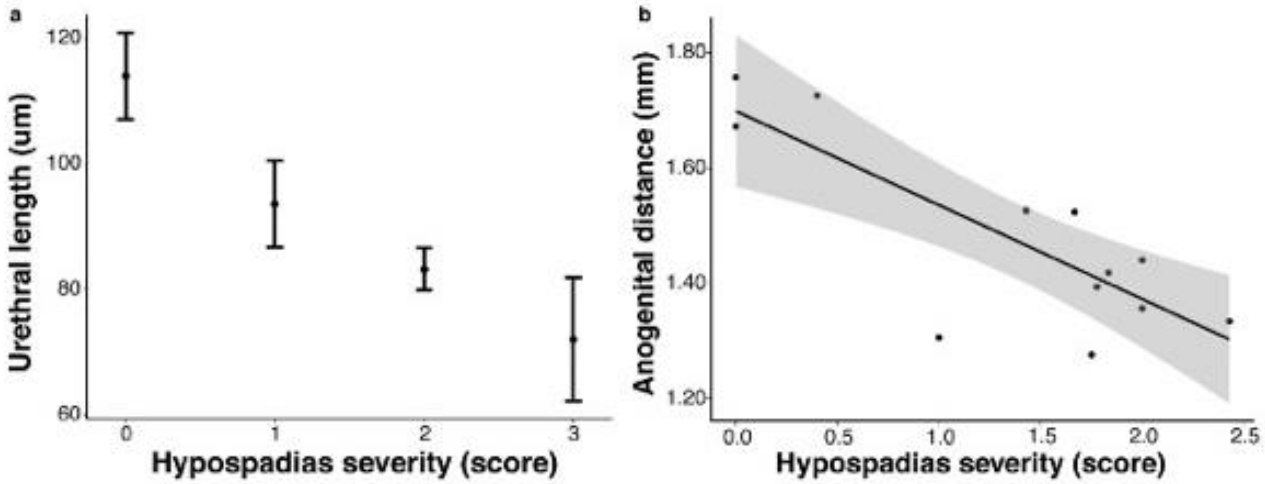


Figure 3. 4 Scoring validation.

a) Urethral length and M.O.U.S.E scores are highly negatively correlated (R^2= .97), b) Average dam score is negatively correlated with anogenital distance (R^2= .6189, p= .001, N=12). N=3 dams/treatment; Score 0 (n=12 pups), Score 1 (n=18 pups), Score 2 (n=37 pups), and Score 3 (n=5 pups). Error bars and error envelope represent 95% confidence intervals.

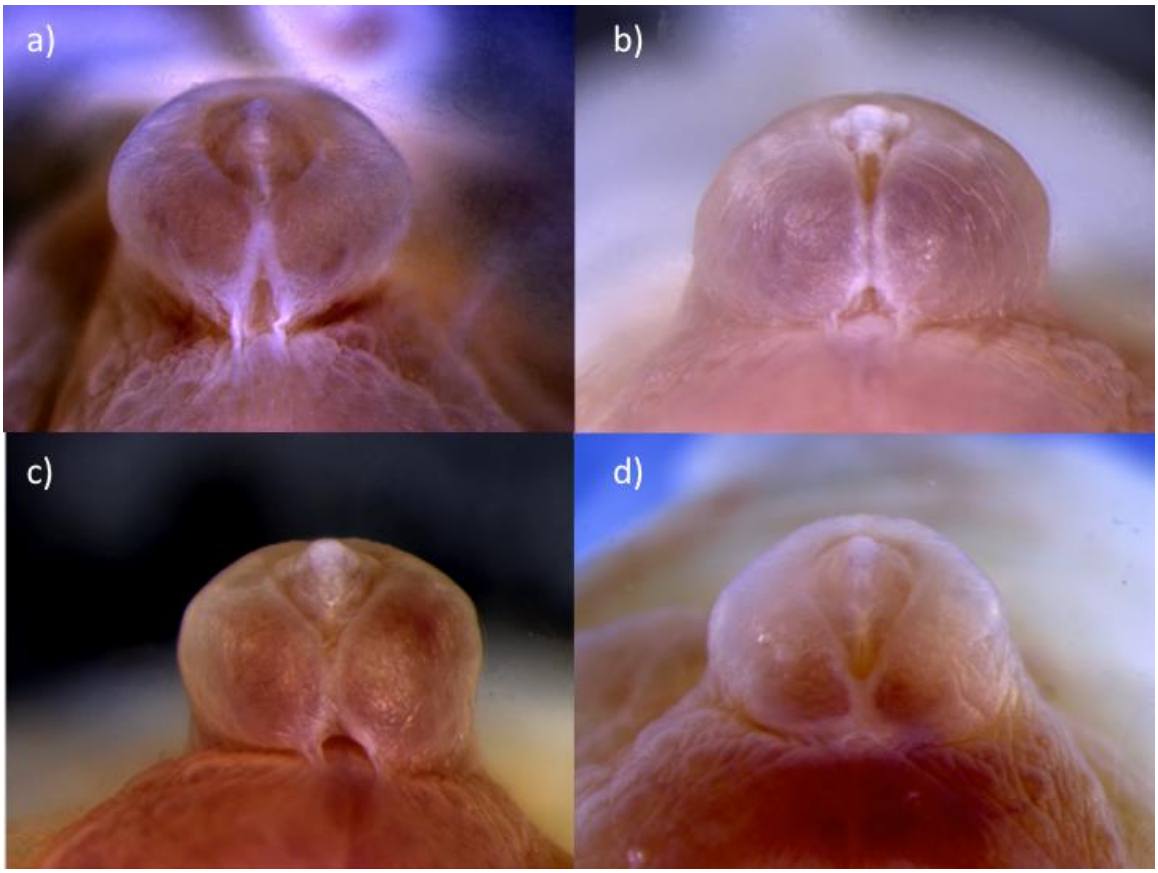


Figure S3. 1Hypospadias severity scores.

a) Score 0- The preputial swellings tightly surround the glans. The urethral exit is distally located, and only a minor ventral cleft is visible at the urethral meatus. Normal genitalia are cylindrical because the height of the dorsal portion of the preputial swellings are similar in height to the ventral portion. In addition the ventral seam appears completely "zipped up". The visible portion of the glans appears circular. b) Score 1- Preputial swellings are more widely spaced at their most ventral and distal extent so the urethra exits further down the ventral side of the genitalia relative to the score of 0 (normal male). Thus the ventral cleft between the preputial swellings is slightly wider and longer relative to individuals scored as 0. The genitalia still has a spherical appearance as the dorsal and ventral preputial swellings are similar in height. The visible portion of the glans is shaped more like an oval as more of its ventral aspect is visible. c) Score 2- The shape of the glans and preputial swellings vary in presentation but in general the

preputial swellings do not wrap completely around the genitalia to seal distally, and the cleft between the swellings is enlarged to form a “V” shape or is deep but narrow. The urethra meatus (bottom of “V” or cleft) is located near the middle of the genitalia. More of the glans penis is visible and can be shaped like an inverted pear (stem of pear forms the point of the “V” shaped cleft). The shape of the genitalia appears less cylindrical because the dorsal region of the preputial swellings that wrap around the glans penis is higher (longer) than the ventral region. d) Score 3- Preputial swelling do not come completely around the genitalia and are only “zipped up” or connected at the most proximal region. Thus the urethral meatus occurs near the base of the genitalia. Much of the ventral glans penis is visible and can be shaped like an inverted teardrop. The shape of the genitalia appears less cylindrical with the dorsal and ventral portions of the preputial swellings being much different in height (as is commonly seen in females). The genitalia phenocopies that of a female

Chapter 4: Organizational effects of the antiandrogen, Vinclozolin, on penis development in the mouse

Abstract

Endocrine disrupting chemicals (EDCs) are pollutants found throughout the environment that disrupt normal endocrine processes. In mice, penis development is thought to be most susceptible to EDCs during a critical developmental period occurring on embryonic days (E) 15.5-17.5. However, androgen signaling begins on E13.5 when Androgen Receptor (AR) protein is found in the genitalia and testosterone is circulating. We hypothesize that disrupting androgen signaling prior to the established critical developmental stage sensitizes the developing penis to future androgen disruption. To test this hypothesis, CD1 dams were exposed to Vinclozolin or a corn oil solvent control on E13.5 and E14.5 and AR levels were measured with immunohistochemistry on E14.5. Early antiandrogen exposure reduced AR within nuclei and decreased intensity of AR expression within E14.5 genitalia. To evaluate the influence of antiandrogen exposure before the known critical stage of penis development, two groups of pregnant dams (n=3) were exposed to Vinclozolin starting at either E13.5 or E14.5 with continued exposure through E16.5. Histology and M.O.U.S.E. scoring were used to quantify penis abnormalities. To account for differences in total doses that mice experienced due to differences in length of dosing time, we compared animals that received the same total doses. Exposure to antiandrogens on E13.5 exacerbated malformations when exposure was continued through sexually dimorphic development. Both exposure time and Vinclozolin dose are important determinants for severity of Vinclozolin-induced penis abnormalities in mice. This

work shows, antiandrogen exposure prior to sensitive periods can exacerbate the effects of later antiandrogen exposure on reproductive development.

Introduction

Endocrine disrupting chemicals (EDCs) are prevalent pollutants in water, air, and soil, and are correlated with increased risk of birth defects across the globe (*105, 115, 165-167*). EDCs are consumed through inhalation, drinking water, skin contact, and food consumption. Many EDCs have estrogenic or anti-androgenic properties that disrupt endogenous sex hormone signaling. Fetal development and sexual differentiation are strongly affected by EDCs, especially when exposure occurs during the critical developmental period during which steroid hormone signaling is known to be active in mediating differentiation of sexually dimorphic organs (*168*). For example, it is well known that, Androgen Receptor (AR) is the apex of a regulatory hierarchy that specifies sexual differentiation of the external genitalia (*169*), but these effects depend on the developmental stage. Mice exposed to high doses of anti-androgenic EDCs during staggered dosing days have different rates of hypospadias incidence (a congenital penis deformity common in humans). Exposure to antiandrogens on embryonic day (E) 15.5 and E17.5 induces the strongest abnormalities in external genital development in mice and rats respectively (*130, 133*). Exposures to EDCs on E13.5 alone or E12.5-E13.5 induce very few hypospadias cases (*25, 133*). Such critical stages of development define when a tissue is most dependent on endocrine signaling and most sensitive to endocrine alterations. The tissues, however, must be organized and made competent to respond to hormones during the critical window. For example, sex steroid signaling organizes the fetal brain to respond to future endocrine signals (*170, 171*). Also, zebrafish developmentally exposed to environmental estrogens show a stronger response to environmental estrogens later in development (*172*). Here, we propose that early antiandrogen exposure, prior to the critical window within which the genitalia is known to be androgen

sensitive, can exaggerate the effects of later EDC exposure by altering AR expression (organization).

In the mouse, external genital development starts on E9.5, and proceeds similarly in both males and females to form a bipotenital genital tubercle (*11*). On E13 fetal Leydig cell differentiation is complete within the testes, and they begin to secrete androgens. These androgens are released into the circulation and bind to ARs in the genital tubercle and initiate a complex cascade of molecular events that masculinize the external genitalia (*117*). These cascades are believed to be the strongest during the critical period within which the genitalia are most sensitive to androgen signaling disruption (e.g., E15.5 in the mouse). AR mRNA, however, has been detected within the genitalia prior to androgen production at E11.5, and protein levels are detectable with immunohistochemistry in the urethral epithelium on the day that androgens are synthesized, E13.5 (*25*). Although, AR protein expression is low at E13.5, with females and males expressing similar amounts of AR (*25*) the necessary elements for androgen signaling are present prior to the critical window. By E15.5 AR is expressed in a sexually dimorphic fashion, and is higher in males than females (*25*), and disruption of AR signaling at this time can "feminize" the genitalia. The molecular mechanisms by which the dimorphism in AR protein quantity becomes established in not well studied.

The development of the penis is a complex process that involves endodermal, mesodermal, and ectodermally derived cells (*7*). All of the resulting tissue types coordinate with each other to induce normal development. Indeed, hypospadias is consistently associated with other de-masculinized phenotypic endpoints (*173*, *174*). In both humans and rodents the developing urethra begins at a location proximal to the tip of the penis and with time the urethral exit migrates up the shaft of the penis as if and open tube is being zipped up (*12*, *175*). In the

rodent model, urorectal septation, proximal urethral opening (PUO), urethral length, and preputial swelling hypoplasia are all associated with urogenital feminization and hypospadias (*11, 12*). In males, the PUO completely closes and the urethral epithelium is septated from the ectodermal epithelium, which results in a tubularized, distally exiting urethra (*13*). As this is happening the preputial swellings expand and wrap around the glans penis. The presence of a PUO in either sex after E16.5 is indicative of abnormal genital development.

Several studies show that the incidence of hypospadias in mice is strongly correlated with antiandrogenic EDC dose, with higher doses resulting in higher hypospadias rates (*114, 131*). The molecular mechanisms underlying the increase in hypospadias severity or controlling variation of hypospadias severity remain elusive. Indeed, hypospadias severity in humans is highly variable (*109*), and understanding the factors that lead to reduced severity will help us develop ways to protect the developing male fetus from abnormal penis development(*129*). Here, we test the hypothesis that blocking androgen signaling, prior to the established critical window of penis development (E15.5-E16.5), but when AR and testosterone are present, leads to disrupted development of the male external genitalia. To test this hypothesis, we used a model antiandrogen (Vinclozolin) to block AR signaling 1 or 2 days before the developmental window within which the genitalia become sexually dimorphic and examined AR levels in the penis and the downstream morphological consequences of this early antiandrogen exposure.

Material and Methods

Mouse maintenance and treatment

All studies were carried out under an approved protocol established by East Carolina University (ECU) Institutional Animal Care and Use Committee (AUP D-297). Eight-week-old CD-1 mice were purchased (Charles River Breeding Laboratories Raleigh, NC) and acclimated

for at least 7 days to 70-72 F on a 12 h light-dark cycle with free access to food and water (Purina ISOCHOW). CD1 mice were time mated by setting 1-2 female mice with a male mouse. Vaginal plugs were checked each morning during the hours of 8:00-10:00 am. The presence of a vaginal plug indicated mating had occurred and the dam was recorded as being embryonic day (E) 0.5 at noon that day. Pregnant dams were removed from the male's cage and were housed with same treatment dams or singingly.

Vinclozolin (Sigma Aldrich, St. Louis, MO, United States,45705), a model anti-androgenic endocrine disrupting pesticide, and its metabolites are known to competitively inhibit the ability of androgen to bind to AR and have been used to consistently induce hypospadias in previous studies (*72, 176, 177*). Vinclozolin is used in this study to block the AR (*129, 151, 178*) to test whether early blockade of androgen signaling alters later AR levels and androgen dependent development. The selected doses were expected to give consistently high rates of hypospadias, but at different severities. The range of penis abnormities allows for severity comparisons within and among the two dose response curves (early vs later exposure times). Vinclozolin doses, 15mg, 20mg, 25mg, or 30mg of Vinclozolin were dissolved in 0.5mL of Tocopherol stripped corn oil (Millipore, Santa Ana, CA, United States, 02901415) for doses 75, 100, 125, 150 mg/kg respectively. The mixture was heated at 60 C and mixed with a Genemate Rotator (H-6800) for 3 hours. Dose volume (µL) received by the pregnant dam was 2.5 multiplied by the weight of the pregnant dam.

Does early anti-androgen exposure affect androgen receptor levels?

Time-mated pregnant dams were orally gavaged with 125 mg/kg of Vinclozolin dissolved in Tocopherol stripped corn oil, or the Tocopherol stripped corn oil solvent control on E13.5 and E14.5 (n_{dam}=3, N_{pup}=6) between the hours of 11:30-13:30. This dose of Vinclozolin is

known to consistently induce severe hypospadias when given during the critical window of development when genitalia become sexually dimorphic (E15-E16) (*129*). One hour after final dose on E14.5 dams were euthanized. Genitalia were collected from male embryos, and briefly washed in 1% phosphate buffered saline (Sigma Aldrich, St. Louis, MO, P3813), then preserved in 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO, United States,158127) overnight at 4 C. Tissues were then submerged in 15% sucrose 2 hours and 30% sucrose overnight, and embedded in OCT (PolarStat; Newcomer supply, Middleton WI, United States, 6405). Genitalia were serially cross-sectioned perpendicular to the proximal to distal axis of the penis at 10 µm. Sections were stored at -80 C until immunohistochemistry procedures were conducted.

Samples were incubated in rabbit anti-Androgen Receptor (1:400; Abcam, ab74272) and rat anti-E-cadherin (1:400; Abcam, ab11512) overnight at 4 C, and then in the secondary goat anti-rabbit 488 (1:400, Abcam, ab150077) and goat anti-rat 647 (1:400, Abcam, ab150167) (Antibody: Supplemental Table 1) antibodies for 1 hour at room temperature and in DAPI (1ug/mL in 1X PBS, ChemCruz, Santa Cruz, CA, United States, sc-3598) for 3 minutes. E-cadherin was used to differentiate the epithelium from the mesenchyme for quantitative analysis. Negative controls (Supplement Figure 1) were labeled and imaged at the same time as the experimental samples. Negative control images are pictures of control males in the ventro-proximal region of the external genitalia where AR concentrations are highest. These negative control slides were not incubated with primary antibodies, but were incubated with the same concentration of secondary antibodies as the sample slides. The tissue was rinsed and washed 3 times between steps with 1X phosphate-buffered saline with 1% Tween (Bio-Rad, Hercules, CA, United States, 1706531) (Supplemental Protocol 1). To eliminate interassay variation, all replicate samples were immunohistochemically processed, imaged (Zeiss LSM 700 confocal

microscope 40x lens; numerical aperture 1.3), and analyzed at one time. For each individual, the mesenchyme at the base of the genitalia one section before the proximal urethral opening (PUO) was identified. Brightness was assessed across all samples with the brightest sample being used to set exposure parameters. All samples were imaged and compared using the same thresholds and exposure times, and since these settings were based on the brightest sample no sample was over exposed.

The observer that collected the AR data was blind to treatment and data was collected from slides in random order. To randomize samples, samples were numerically ordered based on dam and pup number and random numbers were generated using the function (using sample()) in R statistical programing environment), and assigned to each sample in order. Images were then renamed based on their random number assignment, and reordered so that the random numbers were numerically ordered. Each image was then opened in numerical order and a grid of 1.4 x 1.4 µm bounding boxes was generated using the Image J grid function. All bounding boxes located in the mesenchyme were counted and assigned a numerical identifier and random numbers were generated based on the total number of available bounding boxes. Bounding boxes with the numerical identifier that corresponded to the first four random numbers were selected for sampling. For each of the selected bounding boxes the total number of nuclei (DAPI positive), and number of nuclei that were positive for AR (488 positive) were numbered and counted.

Next, to evaluate intensity of AR labeling nine nuclei were randomly selected from the total number of nuclei across all four bounding boxes for each individual. With the numbered nuclei identified, random numbers were generated using sample() for the total nuclei present across all four bounding boxes, and the nuclei corresponding to the first 9 numbers were selected

for evaluation. To evaluate intensity we opened the original czi image file in Zen Blue which was produced during confocal processing using a Zeiss 700 laser scanning microscope. We identified each randomly selected nucleus and measured the intensity of the AR expression using the spline function. Intensity is defined as:

$$Intensity = \frac{\Sigma gray\ values\ in\ 488\ channel}{number\ of\ pixels}$$

Does early anti-androgen exposure affect later penis morphogenesis?

Dose response

To test if blocking early AR signaling prior to the known sensitive window of penis development exacerbates responses to antiandrogens given during the critical window, time mated pregnant dams were assigned to one of two dosing windows, E13.5-16.5 or E14.5-16.5. Fifteen dams were randomly assigned to one of five doses of vinclozolin (n=3) 0, 75, 100, 125 or 150 mg/kg for the first and second dosing windows and were orally gavaged once per day between the hours of 11:30-13:30. We randomized dosing schedules by giving the treatments numerical identifiers, for example, Corn oil = 1, Vinc dose 75mg/kg = 2 etc. then we generated random numbers and assigned dams to treatments based on those numbers. We had a total sample size of 30 dams (5 treatments * 2 dosing windows * 3 replicate dams), and nested within these 30 dams were 151 embryos (see Supplemental Table 2 for more detail).

Day vs. Dose Comparisons

The dosing windows in this experiment were separated by 1 day. This means that E13.5-E16.5 dosing window receives one additional day of treatment. Animals in the same treatment

and longer dosing window (E13.5-E16.5) experienced a higher cumulative dose of vinclozolin. Therefore, any effects ascribed to early exposure could be due to increased total cumulative dose. Our dosing regimen described above provided one dose that could be used to compare total dose across the different dosing windows to test for an effect caused by length of dosing times. Specifically, the 75mg/kg dose over four days is equivalent to 100 mg/kg over three days (300mg/kg). Importantly, however, we also tested if the early exposure (beginning on E13.5) or the cumulative dose was more important at higher doses by assigning an independent set of pregnant dams (N=3) in the E14.5-16.5 dosing window to a 200 mg/kg dose (600 mg/kg total dose) which was compared to the 150mg/kg dose given over the four day E13.5-16.5 dosing window (Table 1). This dose (200mg/kg) was intentionally included in the design so that we had two cumulative doses that overlap which allowed for direct comparisons between time of exposure and total dose. The 200mg/kg dose was left out of the daily dose analyses because it did not overlap with the other doses.

To eliminate variation in developmental stage caused by potential differences in day of birth, dams were euthanized on E18.5 between the hours of 11:30-13:30, fetuses were removed, and the sex of the pups was determined by examining the gonads, which are not affected morphologically by these doses at this particular exposure time window (*129*). Hypospadias presence and severity can be precisely and accurately staged at this time period (*129*). Developmental stage (E18.5) of the embryo was verified by morphological evaluation using the Theiler staging system (stage 26) (*126*). Phenotype of mice did vary within Theiler stage 26, but were never 1 stage greater or less than Theiler stage 26, therefore all mice were considered the same stage for analyses. Genitalia from all mice were removed and stored in 10% neutral

buffered formalin (Richard Allan Scientific, San Diego, CA, United States, 5700TS) until processing.

Histology and morphological data collection

All external genitalia were imaged and the severity of hypospadias was scored using the M.O.U.S.E. protocol (*129*). Genitalia were then dehydrated through a graded series of ethanol baths, cleared with slide bright (xylene substitute; Newcomer Supply, Middleton, WI, United States, ABSB-04), and infiltrated and embedded in paraffin wax (Paraffin X; Newcomer Supply, Middleton, WI, United States, 6534) (*179*) (Supplemental Protocol 2). Genitalia were serially sectioned perpendicular to the proximal to distal axis of the penis (10 μm) and then stained with hematoxylin (Newcomer Supply, Middleton, WI, United States, 1201) and eosin (Newcomer Supply, Middleton, WI, United States, 1072) and microscopically evaluated. A combination of 100x and 200x objectives on the Leica DME microscope was used to identify and define relevant histological landmarks. The length of the genitalia, urethral length, and height of the PUO, and urethra septation height were all collected for analysis (Figure 1). The base of the penis was defined as the first section where the genitalia was disconnected from the perineum, and was established as the first section for data collection. The height of the genitalia was then calculated as the sum of all the sections between the base of the penis and the distal tip multiplied by the section thickness of 10 μm (Figure 1). Likewise, the urethral length was determined by counting the number of sections, from the base to the first section where the urethra opened to the external environment multiplied by section thickness (Figure 1). Next, the presence and distance to the PUO were measured (Figure 1). Septation distance was collected as the number of sections that had a urethra separated from the ventral epithelium of the penis. . Septation width, the space between the centralized urethra and ventral epithelium, was measured on the slide identified as the base of the penis at 200x with a calibrated reticule (Figure 1).

Statistical analysis

All data were analyzed in R statistical programming environment v. 3.4.3 (*180*). Dose response and cumulative dose were analyzed with the lme4 package (*163*). To avoid pseudoreplication, dam was treated as a random effect in all models with pups being nested within dam (*181*). Total number of pups analyzed per treatment x dosing window regimen are displayed in Supplemental Table 2. Assumptions of homoscedasticity, normality, and dispersion were tested by interpreting residual plots, qqplots, and dispersion coefficients, respectively. One dam from the E13.5-16.5, 125 mg/kg group was removed from the analysis due to poor maternal health. The dam underwent gavage stress during the dosing window and lost weight during pregnancy. Pups had severe hypospadias and severe defects in other penis morphological endpoints. They were removed from analysis because they incurred additional stress that pups from other dams did not incur, which we believe exacerbated the effect of Vinclozolin. After outlier removal, this treatment group had a total of 19 and 16 pups (nested in two dams) analyzed for hypospadias quantification and histology respectively.

The mean density of nuclei with AR present and AR intensity were analyzed with a normal error distribution. PUO incidence, septation incidence, and hypospadias incidence was analyzed using a mixed model binomial regression. PUO distance and septation data were analyzed as raw counts of 10 μm histological sections therefore a regression was performed using a Poisson error distribution. A Poisson error distribution was used to phenomenologically describe the relationships between dose, exposure window and the response. Penis length was used to allometrically correct for urethral length as previously described (*129*). Septation width, was collected using the eyepiece micrometer was analyzed with a normal distribution, as the measurements were continuous. Hypospadias severity score (M.O.U.S.E.) proportionally scales with urethral length (*129*) and is on an interval scale. Hypospadias severity was analyzed with a

normal error distribution using linear mixed-models. Nested models included the variables daily dose or cumulative dose, and dosing window and were compared with likelihood ratio tests (LRT) using $p<0.05$ to establish the best model fit (*181*). Cumulative dose was determined by multiplying total days dosed by the daily dose. Daily doses were treated as regression analyses while total doses were analyzed in an ANOVA framework. This allowed us to investigate if severity difference were truly driven by day of exposure, the total dose received, or their interaction. Linear mixed effects models were used for all cumulative dose analyses with dam treated as a random effect. For each response models were compared with LRT using $p<0.05$ to select the best model. Cumulative doses 300 and 600 mg/kg were used to test if embryonic day was significant.

Results

Androgen receptor is diminished by early Vinclozolin exposure

To test if Vinclozolin exposure, prior to the window within which the penis morphogenesis is most sensitive, disorganized AR signaling we used immunofluorescence to quantify the number of cells expressing AR and quantified the intensity of expression. Mice exposed to Vinclozolin on E13.5 and again for one hour on E14.5 ($n_{dam}=3$, $N_{pup}=6$) had diminished density of nuclei expressing AR in the ventro-proximal region of the mesenchyme when compared to corn oil controls (Figure 2C, df=1, $\chi^2=9.1128$, LRT $p=0.000269$) on E14.5. Further those cells that expressed AR in the vinclozolin exposed animals had greatly decreased intensity of nuclear AR expression in the ventro-proximal mesenchyme relative to corn oil controls (Figure 2D, df=1, $\chi^2=13.274$, LRT $p=0.002538$).

Early vinclozolin exposure potentiates continued antiandrogen exposure

To test the developmental implications of pre-critical window reductions in AR nuclear density, we exposed mice to either E13.5-16.5 or E14.5-E16.5 dosing window. No differences in

litter size and mass were observed in any of the treatment groups (LRT p>0.05, Supplemental Table 3). Only sex differences in mass were observed with males being 0.05821 grams more than females (df=1, χ^2=24.0968, LRT p=9.094e-07, Supplemental Table 3). Both dosing windows displayed dose-dependent reductions in masculinization of the external genitalia, but when Vinclozolin exposure began on E13.5 penis morphology was more strongly affected relative to when dosing began on E14.5 (Figure 3, 4, and 5). We found that Vinclozolin exposure beginning on E13.5 led to an interaction between day and dose for hypospadias incidence (n_{dam}=3, N_{pup}=182, Figure 4A, df=1, χ^2=6.9016, LRT p=0.008612) where day of exposure affected the dose response. Both dose and day were significant in the dose response for M.O.U.S.E. (*129*) severity score (n_{dam}=3, N_{pup}=182, Figure 4B, df=1, χ^2=15.7618, LRT p=7.184e-5), but there was not interaction between dose and day. PUO incidence showed an additive change in the dose response (n_{dam}=3, N_{pup}=151, Figure 5A, df=1, χ^2=23.0058, LRT p=1.615e-6). PUO height and septation length showed an interaction between dose and exposure day (n_{dam}=3, N_{pup}=151, Figure 5B, df=1, χ^2=4.1196, LRT p=0.04239) (n_{dam}=3, N_{pup}=151, Figure 5B, df=1, χ^2=12.395, LRT p=0.0004305) and urethral length, corrected for penis length, showed no interaction, but a significant difference in dose and day of exposure in the dose response (n_{dam}=3, N_{pup}=151, Figure 5D, df=1, χ^2=12.5717, LRT p=0.0003916). Depending on the endpoint measured, the timing of exposure either showed an interaction between the dose received and exposure window meaning there was a change in the dose response, or day and dose were significant without an interaction with E13.5-16.5 inducing more severe affects.

Timing of exposure impacts penis abnormalities

To explicitly test whether exposure on day E13.5 or total dose was related to the increased strength of the vinclozolin dose response recorded for the E13.5-16.5 dosing window,

we evaluated penis morphology in individuals that received identical total doses of vinclozolin over the different dosing times (Table 1). Embryonic day of administration significantly impacted penis morphology (Figure 6). Hypospadias severity (n_{dam}=3, N_{pup}=85, Figure 6A, df=1, χ^2=6.7755, LRT p=0.009242), septation height (n_{dam}=3, N_{pup}=73, Figure 6C, df=1, χ^2=6.6149, LRT p=0.01011), and urethral length corrected by penis length (n_{dam}=3, N_{pup}=73, Figure 6D, df=1, χ^2=7.1508, LRT p=0.007493) were significantly more affected when Vinclozolin exposure began on E13.5. Regardless of the dose received, exposure over the period E13.5-16.5 resulted in more severe defects than E14.5-16.5 exposure. Septation (n_{dam}=3, N_{pup}=73, R Script supplement, df=1, χ^2=4.8656, LRT p=0.02740). and PUO incidence (n_{dam}=3, N_{pup}=73, R Script supplement, df=1, χ^2=12.9415, LRT p=0.0003214) both showed day of initial exposure dependent differences. However, hypospadias incidence (n_{dam}=3, N_{pup}=85, R Script supplement, df=1, χ^2=2.3005, LRT p=0.1293) showed no difference for either day or dose of exposure; these doses commonly lead to 100% hypospadias.

The effect induced by Vinclozolin exposure beginning on E13.5 was larger at 300 mg/kg cumulative dose than 600 mg/kg for all endpoints except septation. Animals receiving a cumulative dose of 300 mg/kg and dosed on E13.5-16.5 had 60.26% higher hypospadias severity, 64.33% decrease in septation length, and 13.35% decrease in urethral length relative to those dosed on E14.5-16.5. Individuals receiving a cumulative dose of 600mg/kg on E13.5-16.5 experienced a 22.48% increase in hypospadias severity, 92.86% decrease in septation height, and 8.13% decrease in urethral length relative to those dosed on E14.5-16.5. PUO height showed a significant interaction between cumulative dose and day of exposure (n_{dam}=3, N_{pup}=73, Figure 6B, df=1, χ^2=7.3480, LRT p=0.006714). Animals exposed to a cumulative dose of 300 mg/kg and dosed on E13.5-16.5 had an 89% increase in PUO height relative to those exposed on E14.5-

16.5, whereas animals in the cumulative dose of 600 mg/kg and dosed on E13.5-16.5 showed a 12% increase relative to those dosed on E14.5-16.5.

Discussion

We found that blocking androgen signaling prior to the period within which penis development is most sensitive to antiandrogen exposure reduced nuclear AR localization and intensity (Figure 2), and magnified the genital abnormalities induced by the model anti-androgen Vinclozolin during the androgen sensitive window (Figure 3, 4, 5, and 6). Therefore, in the mouse, the genitalia are organized and made competent to respond to androgens prior to the critical window of genitalia development that defines the most hormonally sensitive period. We believe this potentiation occurs because the autoregulation between AR and circulating androgens is disorganized by early antiandrogen exposure.

Early antiandrogen exposure, prior to the critical window of penis development or hormone sensitive period, changed the dose response of each tissue and made later exposure more potent. For example, lower doses had stronger effects when the animals were exposed on E13.5 (Figure 4 and 5). The increased potency associated with pre-critical window exposure occurs even when animals were exposed to the same total dose. Animals with a cumulative exposure of 300mg/kg had a much stronger response when exposure began on E13.5 (Figure 6) rather than when it began on E14.5. Indeed, the animals exposed to lower cumulative doses showed larger dosing day effects, but even at a total dose of 600mg/kg beginning the dosing regime on day E13.5 consistently lead to stronger effects relative to beginning dosing on E14.5 (Figure 6). The effect of blocking the pre-critical window androgen signaling on E13.5 is not as strong at very high Vinclozolin total concentrations likely because androgen signaling is strongly inhibited during the critical window of genitalia development (E15.5). Our correction of

cumulative Vinclozolin dose assumes that tissue concentrations of Vinclozolin are the same. It is unknown how the single day exposure difference affects Vinclozolin and its metabolite concentrations within penis tissue. The idea that Vinclozolin and it metabolites could be sequestered in penis and potentiate penis defects by displacing androgens and continually binding AR is interesting and requires further investigation.

Although the downstream molecular mechanisms causing the enhanced effects we found need to be investigated, our results demonstrate that tissues must be organized prior to morphological masculinization and that the effects of anti-androgens occur earlier than previously reported. Pre-critical window exposure to Vinclozolin made the mouse fetus more sensitive to EDC exposure during the critical window within which the genitalia become sexually dimorphic. These data suggest that humans' susceptibility to developing hypospadias and the variation in severity of hypospadias cases could be partially explained by timing and dose of anti-androgen exposure.

Understanding the causes of hypospadias severity

Our study showed that severity of hypospadias and related structures involved in penis masculinization were significantly higher when animals were exposed to antiandrogens on the day that androgen signaling is initiated (E13.5). Each day in development, different sets of signals and different regulatory networks must be reconfigured so that the tissues are competent to respond to future signals. As exemplified in this research, the Timing and dosage differences in hormone or EDC exposure can result in different developmental outcomes. In early gential development, E13.5-E15.5, the gross morphology in comparisons between male and female external genitalia remain nearly identical. Our data suggests that during this time androgen signaling is involved in setting up the cells within the urethral epithelium and mesenchyme of the

developing genital primordia to respond differentially to hormonal cues during the critical period of sex-specific genital differentiation. Although morphology is not different, molecular patterns of gene expression are sexually dimorphic at these early stages. We hypothesize that these 'upstream' differences in gene expression and sensitivity to androgens, and possibly estrogens, presage or even play a causal role in the mediation of cell, tissue and organ level developmental differences characteristic of sexually dimorphic genitalia structures.

Developmental-Endocrine signaling

The fact that Vinclozolin exposure induced differences in nuclear AR density within the E14.5 genitalia relative to corn oil controls suggests that antiandrogen exposure diminished androgen dependent upregulation (autoregulation) of AR in the genitalia at E13.5. The later changes in genitalia morphology that we documented suggest that this alteration in positive feedback propagates throughout development. The reduced feedback likely reduced the total AR concentrations and altered the ability of androgens to compete with vinclozolin for AR binding sites. In other words, animals with less AR (e.g. first dosed on E13.5) suffered stronger competitive interactions between vinclozolin (and its metabolites) and androgens for AR. This had profound effects, as individuals exposed beginning on E14.5 required higher vinclozolin exposure to induce similar severities relative to individuals exposed on E13.5 (Figure 4 and 5). Similar autoregulation of AR has been reported in the brain where testosterone exposure increased the density of AR in several different areas of the brain (*182*).

Classically, adult studies have found that blocking androgen signaling results in increased AR levels in several masculine tissues (testis, prostate, brain). In development, we do not observe this, as negative feedback is typically required for homeostasis and not the formation of new organs (*1*). Therefore, developing hypotheses about fetal responses based on information

gleaned from adults can be misleading, as negative feedback regulation must be established before it can function. We show that in the fetal genitalia AR inhibition reduces the quantity of AR and exacerbates the anti-androgen-induced alterations within the external genitalia. This anti-androgen dependent decrease of AR is also found in the Wolffian ducts in fetal rats. For example, flutamide and linuron (anti-androgens) exposure reduced the amount of ARs within the epithelium of the developing ducts (*40, 183*). Sexual dimorphic development culminates in divergent phenotypes but where the morphological outcome lies on the spectrum between fully masculine to feminine genitalia is dependent on timing and dosage of hormone signaling. Positive feedback loops have been identified to cause divergence and induce a bi-stable state leading to two distinct phenotypes (*1*). Here we showed that penis development is influenced by positive feedback of androgen signaling, and early disruption of that feedback results in more severe penis abnormalities. It is likely that other sexually dimorphic organs require similar, positive feedback between androgens and the AR.

Relevance to Human Health

Many reproductive defects, especially in males, have drastically increased over the past 50 years, which is largely thought to be due to the increased prevalence of environmental EDCs (*16, 112, 113, 174, 184-186*). For example, sperm counts are currently decreasing and have declined by 50-60% in Western men since 1973 (*187*). These declines are coincident with environmental factors such as EDC exposure (*165, 188, 189*). In fact, decreased sperm count is commonly associated with birth defects such as cryptorchidism and hypospadias, which suggests that it shares a common prenatal etiology with these reproductive disorders (*106, 187*).

Hypospadias, a birth defect of the penis, increased in incidence by 200% between the years 1970-1990, and now occurs in 1% of boys (*190*) in Colorado and Tennessee, as well as

those born to military families (*191*). In a recent surveillance study, hypospadias was the most common birth defect in 19 (61%) of 31 states surveyed (*191*). In addition to the general increase in hypospadias incidence, severe cases have increased at a faster rate than milder cases (*190*) suggesting that the general increase is not related to better reporting of cases that were commonly missed in the past.

Hypospadias occurs because the urethral folds do not close properly, which causes the urethral opening to occur ventrally along the shaft of the penis rather than at the distal tip. The severity of hypospadias depends on the position of the urethral meatus along the length of the penis, with proximal openings being more severe (*129*). Severe cases of hypospadias result in ambiguous genitalia and in the past led to sexual reassignment surgery for the child (*192*). Corrective penile surgery is an extremely difficult procedure due to different tissue types and the effects of scar tissue on penis function (*107*). Both sexual reassignment and penile reconstruction surgeries place stress on both the babies and their families. Patients with hypospadias have lower self-esteem, and can suffer from difficulty urinating and pain during sexual intercourse (*193*). Identifying the developmental period that organizes is essential because it can explain differences in hypospadias severity in human populations. If we know when the fetus is sensitive to perturbations in the regulatory systems that control normal penal development, we can advise mothers to be especially vigilant about environmental exposures during those times.

Conclusion

In the mouse model early exposure to the anti-androgenic EDC, Vinclozolin affected penis development and overall masculinization of the penis. Early anti-androgen exposure affected the distribution of AR in the penis, and potentiated further endocrine disruption in the genitalia to additional antiandrogen exposure later in development. Ultimately, the interaction of

dosing time, exposure concentration, and genetics must be understood in order to build a synthetic understanding of the origins of hypospadias and which factors determine the degree of the defects that reflect its severity. This synthetic understanding will aid development of preventative therapies for environmentally-induced hypospadias.

Acknowledgments
We would like to thank Dr. Mike McCoy for statistical help and feedback, Josh Mogus for help with dissections, and ECU's veterinary staff for animal husbandry. ECU startup funds to KAM, and the Undergraduate Research and Creative Activities Award to MB were essential for this work.

Supplemental protocol 1

Immunohistochemistry

McCoy Lab

Revised June 2016

General considerations:

- all steps are performed at room temp unless noted

- NEVER let slides dry out once you have rehydrated them; it's best to vacuum off a solution and then immediately add the next one, moving slide by slide through your set of samples

-make enough blocking solution for each of the two days = 4 washes (2 blocks + primary + secondary)--Can store it at 4 °C overnight, after that throw it away

-Make 150uL of block per slide with one slide slop for every 5-10 slides

- the PBS-Triton solutions are good forever on your bench as long as they stay clean (no floaters)

- **optimum dilutions:**
    - **optimum dilutions for most purified antibodies will be between 1:50-1:50**

- **optimum dilutions for most ascites antibodies will be between 1:5-1:10**
- **most AlexaFluor secondaries should be diluted at 1:400,**
- **most Jackson ImmunoResearch secondaries at 1:200**

-any wash ≤ 30 min should be done with the lid on

- after the secondary antibody has been added, every subsequent wash needs to be in the dark

---

Solutions:

PBS-autoclaved

PBS-Tw: 0.1% + Tween-20 in PBS

[500ul Tween-20 in 500mL PBS]

** if using less concentrated tween adjust volumes accordingly

0.5% Triton: 0.5% Triton X-100 in PBS

[250uL Triton + 50mL (- 250uL) PBS]

BLOCK--PBS-Tw-B-GS: 2% lyophilized bovine serum albumin (B) + 10% goat serum (G) in PBS-Tw

[0.2 g bovine serum albumin + 1mL GS + 9mLPBS-Tw]

**can keep in the refrigerator overnight or a few days max-- make sure you see no precipitation when you use it—vortex if precipitate still there toss and make new

Dapi (sc-3598): 1ug/ml in PBS

[1ul of 1mg/mL stock into 999ul PBS] need 150uL/slide plus slop

Primary Antibodies: 150uL/slide + slop

1)Rabbit anti-Androgen Receptor IgG1 @1:400, abcam, 06-570 diluted in PBS-Tw-B-GS

2)Rat anti-E-cadherin IgG1 @1:400, abcam, 06-570 diluted in PBS-Tw-B-GS

Secondary Antibodies (check dilution): 150uL/slide + slop

1) Goat anti-rabbit IgG AlexaFlour 488 @1:400 diluted in PBS-Tw-B-GS

2) Goat anti-rat IgG AlexaFlour 647 @1:400 diluted in PBS-Tw-B-GS

Calculation work space:

Total volume needed (ul) divided by dilution denominator (e.g., for AR above 400)= uL of primary needed for your total volume

Day 1 (~2 hours)

a) Prepare all solutions (e.g., block, and primaries); aliquot as needed___
b) If block and antibody were prepared previously, take out of refrigerator and bring to RT___
c) Remove slides from -80 °C and thaw to room temp, allow any condensation to dry __
d) Pap pen around sections & allow to dry __
e) Place slides in humidity box with paper towels wetted with PBS at bottom __
f) Rehydrate in PBS-Tw 10 min___
g) Premeabilize in 0.5% Triton for 30 min ___
h) Rinse 2x in PBS-Tw ___ ___
i) Wash in PBS-Tw 3 x 5min ___ ___ ___
j) Mix PBS-Tw-B-GS solution well, block 20 min. in PBS-Tw-B-GS ___
k) Incubate overnight at 4°C in primary antibody ___ (negative gets block only)
If multi-labeling, make sure that the species each primary is raised in are different
l) Place leftover block and antibody in refrigerator___

DAY 2 (~2.5 hours)

m) Prepare all solutions (e.g., block, and secondaries); aliquot as needed___
n) If block and antibody were prepared previously, take out of refrigerator and bring to RT___
o) Remove slides from refrigerator
p) Rinse 3x in PBS-Tw ___ ___ ___
q) Wash in PBS-Tw 4 x 5min ___ ___ ___ ___
r) Mix PBS-Tw-B-GS solution well, block 20 min. in PBS-Tw-B-GS ___
s) Incubate secondary antibody diluted in PBS-Tw-B-GS @ RT 1 hour ___
t) Rinse 2x in PBS-Tw ___ ___
u) Wash in PBS-Tw 3 x 5min ___ ___ ___
v) Mix dapi well, incubate in dapi 3 min___
w) Rinse 2x in PBS-Tw ___ ___
x) Wash in PBS-Tw 3 x 5min ___ ___ ___
y) Mix mounting medium well, mount with mounting medium (DAKO fluorescent mounting medium or ___
z) Store at RT overnight to dry in dark but open area
aa) Place leftover block and antibody in refrigerator___

DAY 3 (~15 min)

bb) seal slides with finger nail polish ___
cc) Store in dark at 4° C (preferably bottom shelf) ___

Supplemental protocol 2

Histology Protocol

Processing:

Machine-Tissue TEK VIP 2000 Processer

Operating Procedure

70% Ethanol -	2 hours 24C	Slot 1
80% Ethanol-	1 hour 24C	2
95% Ethanol-	1 hour 24C	3
95% Ethanol-	1 hour 24C	4
100% Ethanol-	1 hour 24C	5
100% Ethanol-	1 hour 24C	6
100% Ethanol-	1 hour 24C	7
Slide Brite-	1 hour 24C	8
Slide Brite -	1 hour 24C	9
Slide Brite -	1 hour 24C	10
Ultraffin X-	1 hour 55C	11
Ultraffin X-	1 hour 55C	12
Ultraffin X-	2 hours (under vacuum) 55C	13

Embed in Ultraffin X

Staining

Preparation

1) Transfer solutions from holding containers to staining dishes

a. Run hematoxylin through a filter in a funnel (oxidizing)

2) Change solutions based on the solution changing sheet
3) Check the solution volume, make sure slides will be completely submerged in the solution
4) Have timers on hand
5) Place slides in slide holders and begin…

5□m Sections

Slide Bright	3 minutes
Slide Bright	3 minutes
Slide Bright	4 minutes
100% Ethanol	5 minutes
100% Ethanol	5 minutes
95% Ethanol	3 minutes
95% Ethanol	3 minutes
80% Ethanol	3 minutes
dH_2O	3 minutes
Harris Hematoxylin	4 minutes
Running tap water	5 minutes
1% acid ethanol	1 dip
Tap water	1 dip
Scotts Tap Water	2 dips
Running tap water	10 minutes
80% Ethanol	2 minutes
Eosin-Phloxine	2 minutes
95% Ethanol	2 minutes
95% Ethanol	1 minutes
100% Ethanol	2 minutes

100% Ethanol	2 minutes
Slide Bright	3 minutes
Slide Bright	3 minutes
Slide Bright	3 minutes
Coverslip with Permount	

RESULTS:

Nuclei	Blue
Cytoplasm	Pink or red
Most other tissue structures	Pink or red

Tables

Table 4. 1 Calculated cumulative doses for each window

Dosing Window	Number of Exposure Days	Daily Dose (mg/kg/day)	Cumulative Dose (mg/kg)
E13.5-E16.5	4	75	300
	4	150	600
E14.5-E16.5	3	100	300
	3	200	600

Table S4. 1 Details of antibodies used for immunohistochemistry

Protein	Host Species	Type Antibody	Dilution	Catalog Number
Androgen Receptor	Rabbit	Polyclonal	1:400	ab74272

E-Cadherin	Rat	Monoclonal	1:400	ab11512
Anti-rabbit 488	Goat	Polyclonal	1:400	ab150077
Anti-rat 647	Goat	Polyclonal	1:400	ab150167

Table S4. 2 Weight and litter size mean and standard error for dose responses

Dosing Window	Daily Dose(mg/kg/day)	Sex	Mass (grams)	Pups/Litter
E13.5-E16.5	0	Male	1.3870 ± 0.1023	5.00 ± 0.5774
		Female	1.3587 ± 0.0659	9.33 ± 0.5774
	75	Male	1.4865 ± 0.0933	7.67 ± 1.4530
		Female	1.4003 ± 0.1252	7.00 ± 0.0000
	100	Male	1.5008 ± 0.0381	5.33 ± 1.2019
		Female	1.4371 ± 0.0190	5.67 ± 1.2019
	125	Male	1.2771 ± 0.0015	9.50 ± 0.4082
		Female	1.2343 ± 0.0060	4.00 ± 0.8165
	150	Male	1.3747 ± 0.0359	8.00 ± 0.5774

		Female	1.3258 ± 0.0192	6.33 ± 0.3333
E14.5-E16.5	0	Male	1.4511 ± 0.0882	5.67 ± 1.7638
		Female	1.3876 ± 0.0910	9.33 ± 2.0817
	75	Male	1.4222 ± 0.0563	6.67 ± 1.2018
		Female	1.3876 ± 0.0721	8.67 ± 1.2018
	100	Male	1.3805 ± 0.0133	5.67 ± 2.0276
		Female	1.3254 ± 0.0491	5.33 ± 1.2019
	125	Male	1.3524 ± 0.0569	5.67 ± 1.2019
		Female	1.3026 ± 0.0503	5.67 ± 1.3333
	150	Male	1.3391 ± 0.0357	4.67 ± 0.5774
		Female	1.2544 ± 0.3807	8 ± 2.0817
	200	Male	1.3910 ± 0.0793	5 ± 0.5773
		Female	1.3246 ± 0.0799	9 ± 1.4530

Table S4. 3 Total pups analyzed for each treatment X dosing window regimen

Dose	Day	Total Pups Evaluated for Hypospadias Severity	Total Pups Histologically Analyzed
0	13	15	14
0	14	17	12
75	13	22	18
75	14	17	14
100	13	15	15
100	14	26	22
125	13	19	16
125	14	17	10
150	13	23	19
150	14	14	11

Figures

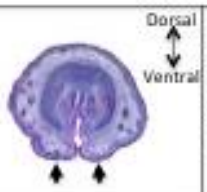
Urethral Exit		First section where the epithelium of the two opposing preputial swellings (black arrows) are disconnected on the ventral side.
Urorectal Septum End		Last section where the urethra (white arrow) is separated from the ventral aspect of the genitalia by mesenchyme (black arrow).
Proximal Urethral Opening		Two opposing preputial swellings (black arrows) are disconnected on the ventral side. The first and last section that contained the proximal urethral opening was collected.
Tubercle base		First section where the external genitalia epidermis was separated from the perineum epidermis (black arrow).

Figure 4. 1 Morphological landmarks for data collection

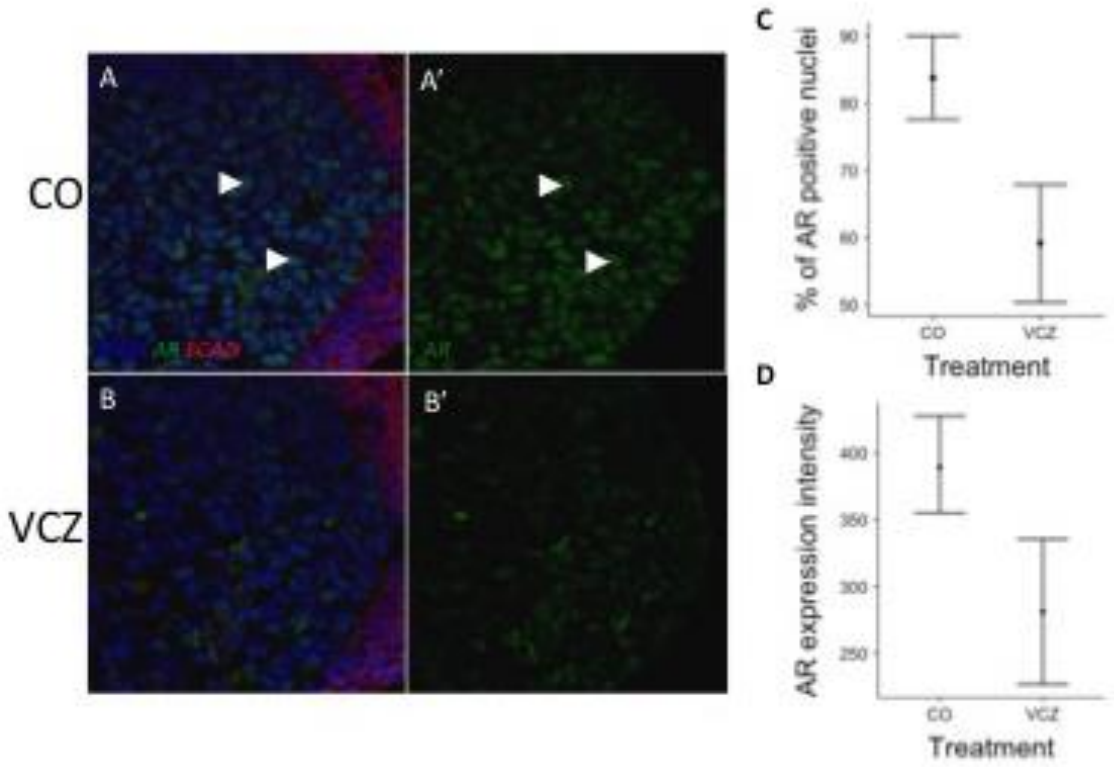


Figure 4. 2 Vinclozolin exposure on E13 and E14 diminishes AR expression.
A and **A'**) Control animals have more nuclear expression of AR at higher intensities than vinclozolin treated animals **B** and **B'**. **C** and **D**) quantification of of AR expression intensity and cell density. Error bars represent 95% confidence intervals derived from model estimates. n_{dam}=3, N_{pup}=6. * indicates p-value < 0.05.

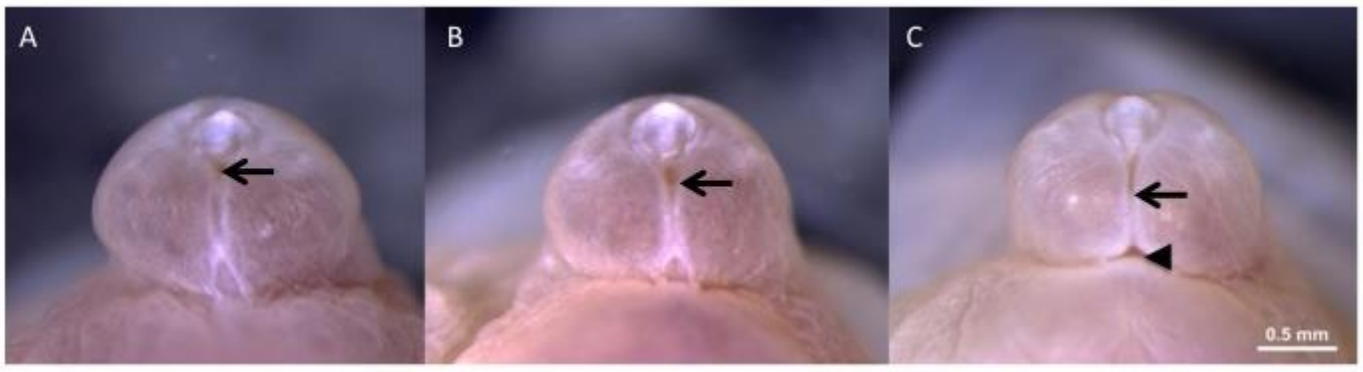


Figure 4. 3 External morphology differs at the same doses.

A) Control animals have a distally exiting urethra (black arrow) and lack a PUO. **B)** Fetuses exposed to 75 mg/kg of Vinclozolin on E14.5-16.5 have urethral exits very close to the distal tip and little to no PUO. **C)** Fetuses exposed to 75 mg/kg of Vinclozolin on E13.5-16.5 have urethra exits at mid-shaft and have a large PUO (black arrowhead).

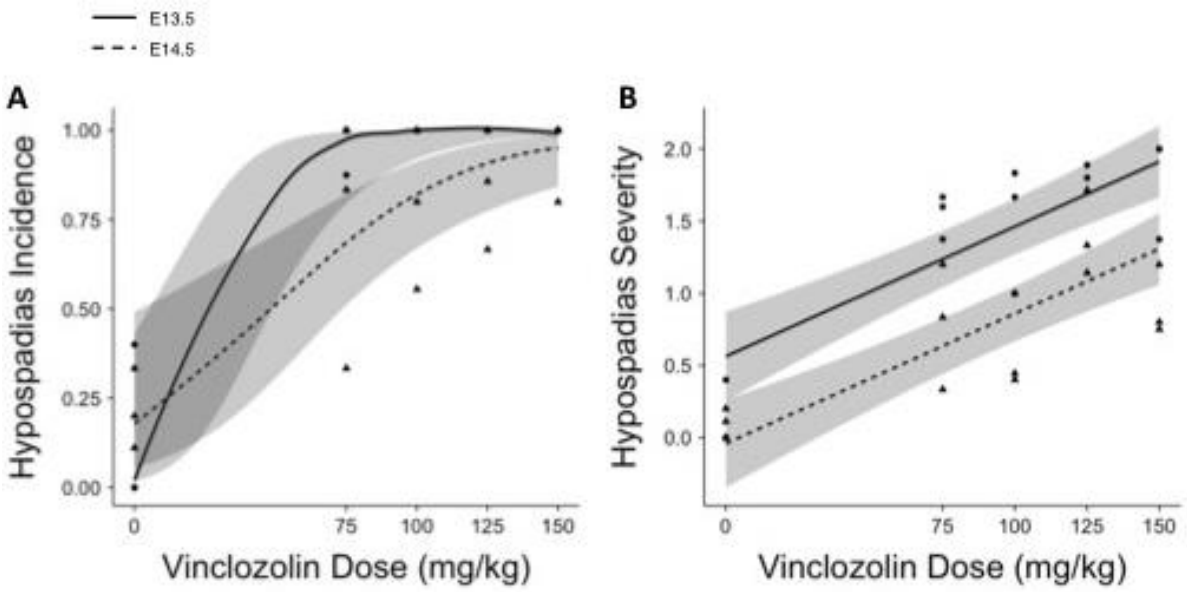


Figure 4. 4 E13 Vinclozolin exposure induces higher rates and more severe hypospadias.
A) Hypospadias rates showed a significant interaction between day of exposure and dose received (different slopes). In the E13.5-16.5 (solid) the incidence of hypospadias reached 100% between doses 75 mg/kg and 100 mg/kg , while hypospadias rates never saturate at 100% in the E14.5-16.5 dosing window (dashed). **B)** The dose response for hypospadias severity showed a significant difference between E13.5-16.5 and E14.5-16.5 dosing windows. E13.5-16.5 had significantly more severe hypospadias than E14.5-16.5 dosing window. Circle points signify dam means for 13.5-16.5 dosing window and triangle points signify dam means 14.5-16.5 dosing window. Binomial and linear regressions were used for hypospadias incidence and severity data, respectively. Likelihood ratio tests were used to select the best model and a p-value < 0.05 was

used to assess significant parameters in the model. Shaded envelopes are 95% confidence intervals derived from model estimates, $n_{dam} = 3$, $N_{pup} = 182$.

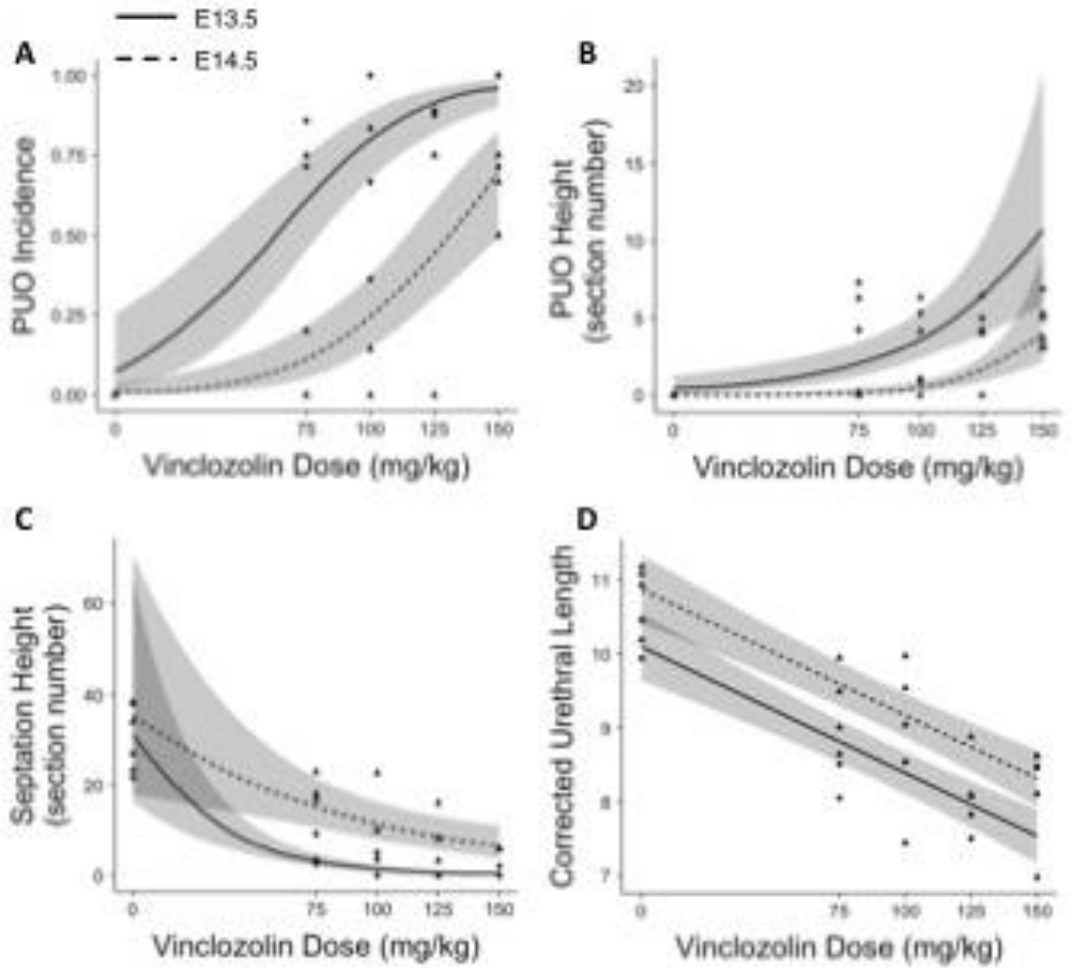


Figure 4. 5 Vinclozolin exposure on E13 causes more severe penile abnormalities.
A) PUO incidence showed significant differences where rates reached nearly 100% in the E13.5-16.5 (solid) dosing window whereas the E14.5-16.5 (dashed) dosing window did not induce this level of PUOs. **B and C)** PUO height and septation showed a significant interaction between day of exposure and Vinclozolin dose. Both endpoints showed vinclozolin to be more potent in E13.5-16.5 dosing window compared to E14.5-16.5. **D)** Urethral length had a significant difference where the E13.5-16.5 dosing window led to smaller urethras relative to E14.5-16.5 .

Circle points signify dam means for 13.5-16.5 dosing window and triangle points signify dam means 14.5-16.5 dosing window. Binomial regression was used for PUO incidence data, Poisson regression was used for PUO and septation data and linear regressions was used for urethral length data. The penis by urethral length allometric relationship was used to correct urethral length for the length of the penis (urethral length scales as a power function of 0.34061 of penis length). Likelihood ratio tests were used to select the best model and a p-value < 0.05 was used to assess significant parameters in the model. Shaded envelopes are 95% confidence intervals derived from model estimates, $n_{dam} = 3$, $N_{pup} = 151$. All measurement data is presented as number of sections, to obtain metric sizes multiple sections by 10 μm.

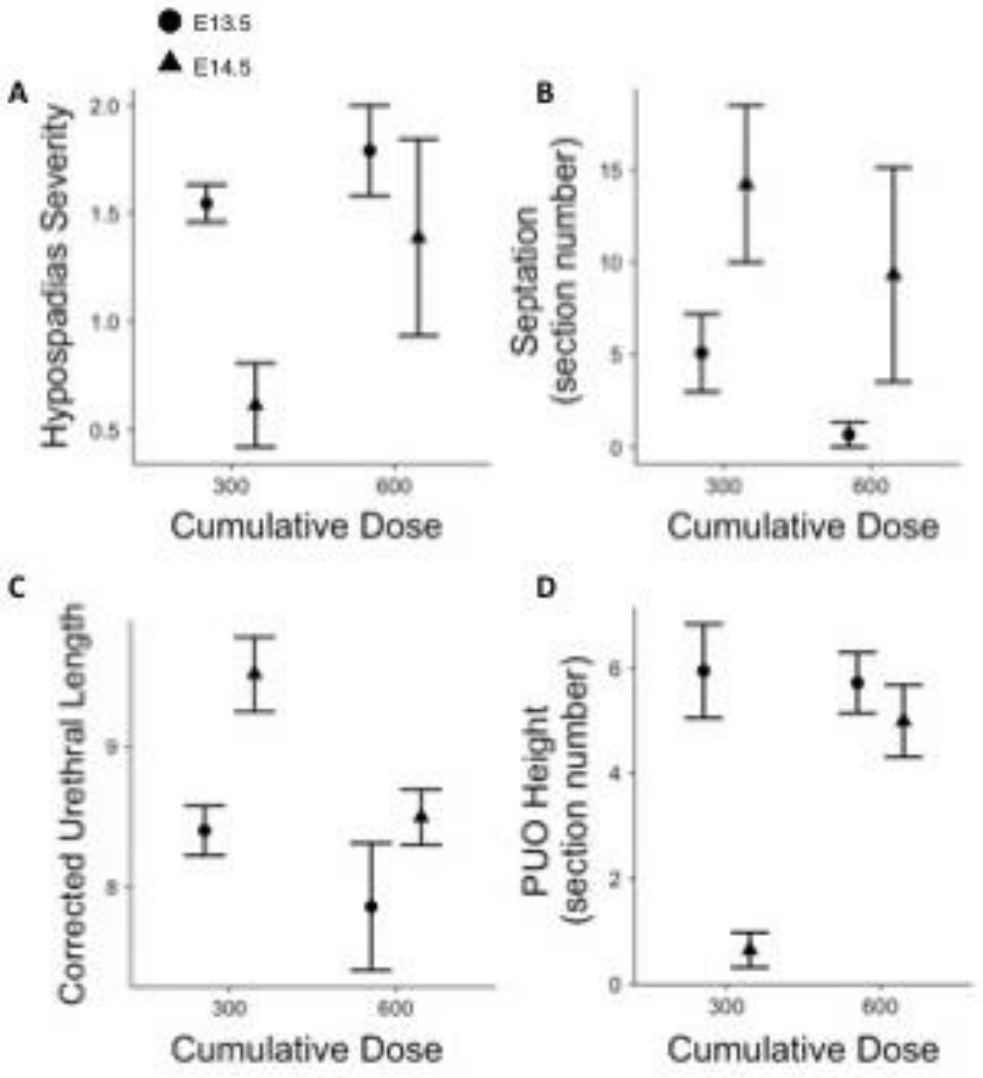


Figure 4. 6 Vinclozolin exposure on E13.5 induces stronger effects.

A) With 300 and 600 mg/kg total dose the severity of hypospadias was significantly greater in E13.5-16.5 when compared to E14.5-16.5 treated fetuses. E13.5-16.5 (circle) had more severe hypospadias than E14.5-16.5(triangle) dosing window for both doses. **B)** At 300 mg/kg total dose of Vinclozolin E13.5-16.5 showed larger PUOs than E14.5-16.5 animals. At 600 mg/kg there was no difference in PUO height due to day of exposure. **C and D)** Septation height and urethral length both show a significant effect of dosing window exposure**.** The penis by urethral length allometric relationship was used to correct urethral length for the length of the penis

(urethral length scales 0.34061 of penis length). Error bars represent standard error derived from the raw data. $n_{dam} = 3$, $N_{pup} = 73$. All measurement data presented as number of sections, to obtain metric sizes multiply sections by 10 μm.

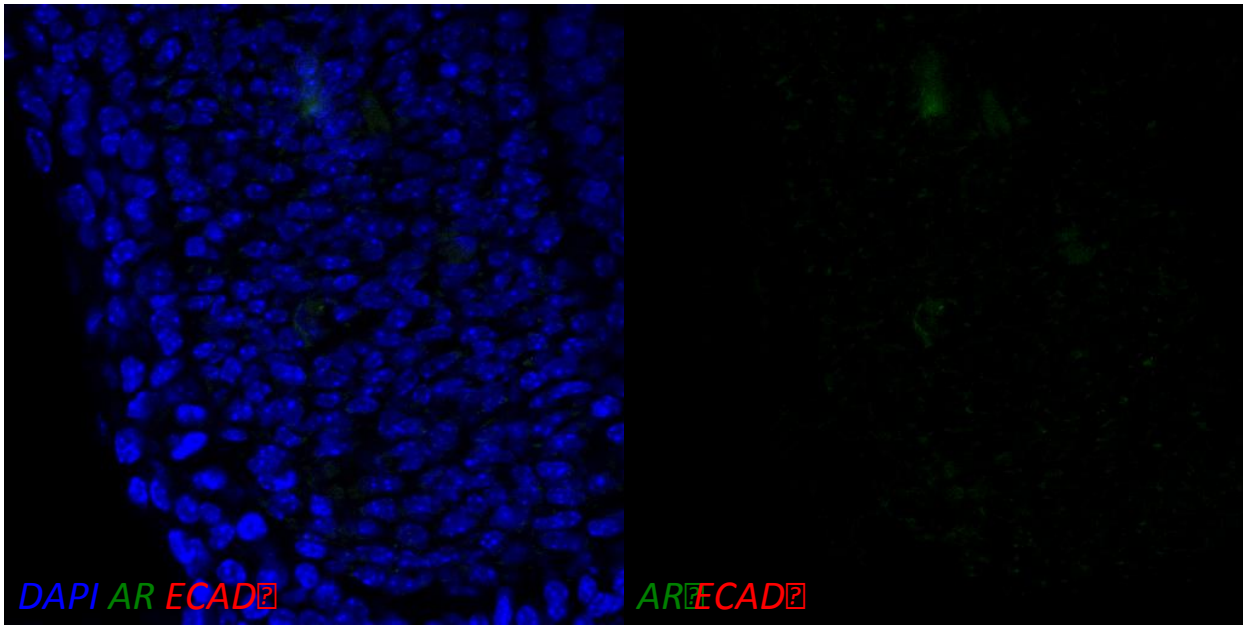


Figure S4. 1 Negative Control.
Control male sample in the proximo-ventral region of the external genitalia that did not receive primary antibody for Androgen Receptor and E-Cadherin, but did receive secondary antibodies.

Chapter 5: Mitigating EDC-induced hypospadias with the NFE2L2 activator, sulforaphane

Abstract

Pollutant exposure is unavoidable and induces many health problems including birth defects. Genes like, Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2), have evolved to detoxify natural toxins and reduce toxin-induced oxidative stress. It has been proposed that sulforaphane, a potent activator of NFE2L2, could, therefore, reduce the impact of embryonic toxicant exposure. To test this hypothesis we exposed pregnant CD1 mice to the anti-androgen Vinclozolin (125 mg/kg) and one of eight doses of sulforaphane (0-120mg/kg), and measured anogenital distance (AGD) (a biomarker of masculine development) and hypospadias severity (a model pollutant-induced birth defect). We found that 45 mg/kg of sulforaphane induced the greatest rescue in AGD and reduction in hypospadias severity. To test if sulforaphane could decrease pollutant potency we dosed CD1 mice to either 0 or 45 mg/kg of sulforaphane and one of five doses of Vinclozolin (0-125mg/kg). Sulforaphane treatment significantly reduced the negative effects of vinclozolin and decreased hypospadias incidence, severity, and increased biomarkers of masculinization (e.g., urorectal septation). Finally, to test if the sulforaphane-induced rescue required NFE2L2, we mated and dosed $NFE2L2^{+/-}$ BL6 mice to produce $NFE2L2^{+/+}$ and $NFE2L2^{-/-}$ embryos. We dosed dams with a corn oil control, sulforaphane (45 mg/kg) only, vinclozolin (100 mg/kg) only, or Vinclozolin (100 mg/kg) + sulforaphane (45 mg/kg). $NFE2L2^{+/+}$ showed a significant sulforaphane-induced reduction in hypospadias severity and increase of AGD whereas $NFE2L2^{-/-}$ did not. Sulforaphane reduced the impacts of pollutant exposure during embryonic development and requires NFE2L2 to rescue normal developmental pathways.

Introduction

Environmental pollutants are ubiquitously distributed in soil, water, and the atmosphere, and most humans are exposed to toxicants throughout life via ingestion, inhalation or dermal contact (*194*). These

seemingly inevitable exposures can perturb several physiological and developmental processes. For example, endocrine disrupting chemicals (EDCs) are environmental contaminants that disrupt many aspects of endocrine signaling and regulation, which induces malformations and various pathologies (*100, 115, 195*). Embryonic exposure to EDCs has a severe effect on the developing fetus because their organs are in the process of differentiating and are in a state of strong genetic and hormonal responsiveness, which environmental chemicals can perturb (*100, 196*). Physiological dysregulation during critical developmental periods can have negative health implications that transcend into adulthood and across a lifespan (*197*). Several congenital malformations of the reproductive system, and adult diseases, such as cardiovascular disease, and obesity have been linked to embryonic exposure to environmental pollutants such as endocrine disrupting chemicals (EDCs) (*198, 199{Bernal, 2010 #43, 200*). Reducing these effects is highly desirable. Given, the prevalence of environmental pollutants, however, it is unrealistic to believe that we can fully protect ourselves or the fetus by preventing exposure.

Recently, it was proposed that sulforaphane, a nutrient obtained from cruciferous vegetables such as broccoli, could be developed as a general preventative agent to protect against toxicant exposure(*201, 202*). Sulforaphane is a strong activator of Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2), which is a transcription factor that upregulates detoxification and antioxidant proteins (*202, 203*) (Figure 1). Typically, NFE2L2 is held quiescently in the cytosol bound to Keap1 by 4 sets of disulfide bridges (*204*). Under oxidative stress the disulfide bridges are broken, which allows NFE2L2 to translocate to the nucleus and transcribe a number for Phase I and Phase II detoxification enzymes (*204*). Sulforaphane breaks the disulfide bridges between Keap1 and NFE2L2 and thus induces NFE2L2 dependent transcription (*205*). Activation of NFE2L2 has previously been shown to reduce the negative impacts of air pollution{Egner, 2014 #67}, phthalate exposure (*206*), and chemotherapy (*207*). To our knowledge, however, sulforaphane has not been tested as a prenatal supplement to protect the developing fetus from toxicant exposure. Indeed, the development of a prenatal supplement to help buffer the developing fetus

from environmental pollutants would be invaluable. Here, we take the first critical step to develop such a therapy.

One common birth defect associated with prenatal exposure to EDCs is hypospadias, a congenital abnormality of the penis. Hypospadias occurs when the urethra does not open at the distal tip of the penis but rather when it opens ventrally along the shaft (*107*). Hypospadias is one of the most common birth defects in the United States occurring in up to 1% of male newborns each year (*208*). The incidence of hypospadias has approximately doubled from the 1970s to the 1990s with more severe cases increasing at a faster rate than mild cases (*190*). Hypospadias is now the most common birth defect in 19 of the 37 US states that were recently surveyed (*107, 208*). Surgical correction of the external genitalia is challenging due to the amount of soft tissue and excessive scar tissue that interferes with urethral function (*209*). Even after corrective surgery patients often experience painful erections, reduced fertility, difficulty urinating, and can have lasting negative psychological effects (*210*).

EDC-induced hypospadias is an excellent model contaminant-induced birth defect to use to study protective therapies because it is a common birth defect in humans, provides a quantifiable phenotype, and can be induced in laboratory mice. For example, we can induce hypospadias 100% of the time in the mouse using the model anti-androgenic EDC, Vinclozolin (*128*). We have also developed a standardized scoring system that allows precise and accurate evaluation of hypospadias severity (*129*). This dosing regimen and scoring system facilitate evaluation of preventative therapies as we can quantitatively define if the incidence and severity of the birth defect is reduced by nutritive supplements.

Using this validated model, we test the dose response relationship of sulforaphane in animals exposed to very high concentrations of Vinclozolin, and identify the dose of sulforaphane that most strongly mitigates the demasculinizing effects of this anti-androgen. Using, the most effective dose of sulforaphane, we then determine the extent to which it can reduce the potency of the Vinclozolin dose response. Finally, we test the hypothesis that sulforaphane requires NFE2L2 to induce its protective

benefits. Indeed, we find that sulforaphane induces strong protection against Vinclozolin and that those benefits depend on the presence of the highly conserved, protective transcription factor NFE2L2.

Results

Sulforaphane protects the fetus from high antiandrogen exposure

To test the hypothesis that sulforaphane can restore masculine development in Vinclozolin-exposed individuals, we exposed mice to 125 mg/kg of Vinclozolin from embryonic day (E) 13.5-16.5, which induces 100% genital abnormalities (*128*), and one of eight doses of sulforaphane. We measured the size of the anogenital distance with an eyepiece micrometer and scored the severity of hypospadias using the standardized M.O.U.S.E. scoring system (*129*). Sulforaphane induced a non-monotonic change in anogenital distance. There was a dose-dependent increase in anogenital distance (masculinization) through 60 mg/kg of sulforaphane exposure (Figure 1A p=0.002902). At the highest point of rescue (45mg/kg) we observed at 13.95% increase of anogenital distance relative to the no sulforaphane (vinclozolin only) treatment. At 75 mg/kg of sulforaphane, the sulforaphane-dependent rescue of anogenital distance was similar to that of the Vinclozolin only treatments with only a 3.76% increase in AGD relative to the Vinclozolin only treated animals. 120mg/kg of sulforaphane further reduced anogenital distance and only lead to a rescue of 1.4%.

Hypospadias severity, measured using standardized M.O.U.S.E scores (*129*) also displayed a nonmonotonic dose response to sulforaphane. Hypospadias severity showed a linear rescue (decrease) across sulforaphane doses in until 60 mg/kg of sulforaphane supplementation. Again, 45 mg/kg of sulforaphane induced the greatest reduction in hypospadias severity, with a 32.54% reduction in severity score. At 75 and 120 mg/kg of sulforaphane supplementation, however, hypospadias severity was increased to Vinclozolin only levels.

At 45 mg/kg sulforaphane treatment results in partially ‘normal’ anogenital distance and penis development. When compared to corn oil controls, vinclozolin only (0 sulforaphane) exposure induced a 31.2% decrease in anogenital distance, which shows that sulforphane abrogated the deleterious effects of

vinclozolin; anogential distance by 9.6%. Sulforaphane + Vinclozolin exposure anogenital distance is reduced by 21.6%. For hypospadias severity, scored by M.O.U.S.E. Vinclozolin only treatment induced a large effect; with corn oil scores being 0.055 and the Vinclozolin only score being 1.69 (Figure 1B). Vinclozolin + sulforaphane exposure resulted in a mean severity score of 1.14 which is a reduction of 32% in the severity of normal penis development even when embryos are exposed to very high doses of the anti-androgen Vinclozolin.

Sulforaphane reduces the Vinclozolin dose response

To test if Sulforphane decreases the potency of Vinclozolin, we exposed pregnant CD1 mice to either 0 or 45 mg/kg of Sulforaphane and five doses of Vinclozolin. Both Vinclozolin-only and sulforaphane + Vinclozolin treatment displayed dose dependent effects with Vinclozolin dose. There was, however, a significant interaction between sulforaphane presence and vinclozolin dose where the slope of the vinclozolin dose response was shallower (reduced) in the presence of sulforaphane. Both hypospadias incidence (Figure 2A, df=1, χ^2=7.6019, LRT p=0.005381) and hypospadias severity (Figure 2B, df=1, χ^2=4.8313, LRT p=0.027948) were reduced across all doses of Vinclozolin when sulforaphane was also supplemented.

We went further to investigate additional androgen-dependent aspects of penis morphology to determine whether sulforaphane was generally protective and could decrease the potency of Vinclozolin across tissues types and developmental processes. For example, proximal urethral opening (PUO) height and incidence were measured. In females, PUO is reconstructed into the vagina but is very small or non-existent at E18.5 and males completely lack a PUO at E18.5 (*12, 57, 128, 211*). In males the PUO is closed by mesenchymal infiltration and septation of the urethral plate (*12, 211*). Normally the mesenchymal infiltration extends to the distal tip of the penis and pushes the urethra into the middle of the penis. Antiandrogen exposure disrupts this process and can result in complete absence of mesenchymal infiltration (*13*).

Males exposed to Vinclozolin only in this study had high incidences and large PUOs when compared to control males (*129*). The presence of this abnormal opening resulting from Vinclozolin treatment was completely eliminated at all but the highest Vinclozolin dose (Figure 2C). In the presence of 45mg/kg of sulforaphane none of the embryos exposed to 50, 75, 100, or 125 mg/kg of Vinclozolin had PUOs, while 50% of the 50 mg/kg and 100% of the 75 and 100 mg/kg Vinclozolin exposed embryos had PUOs. PUO height was significantly reduced with sulforaphane supplementation (Figure 2C, df=1, χ^2=17.4581, LRT p=2.937 x 10^{-5}) relative to Vinclozolin and no sulforaphane supplementation.

The effects of Vinclozolin on mesenchymal infiltration and septation of the urethral plate epithelium showed a significant interaction between sulforaphane and Vinclozolin dose (Figure 2D, df=1, χ^2=16.704, LRT p=4.368x10^{-5}). Sulforaphane exposure resulted in higher, more normal mesenchymal infiltrations. At 50 mg/kg of Vinclozolin septation heights were very similar to that of sulforaphane + vinclozolin and control exposures, at 75 mg/kg there was an increase in septation size with sulforaphane presence when compared to vinclozolin only. This 45 mg/kg of sulforaphane rescue of septation height persisted from 75 – 125 mg/kg Vinclozolin doses.

NFE2L2 is required for sulforaphane-dependent rescue

Sulforaphane is a potent activator of the transcription factor, NFE2L2 (*212*). To test if the rescue that we consistently observe requires the presence of NFE2L2 we crossed male and female NFE2L2+/- heterozygotes to produce wild type (NFE2L2$^{+/+}$) and null allele (NFE2L2$^{-/-}$) embryos, and exposed pregnant dams to corn oil only, 45 mg/kg of sulforaphane, 100 mg/kg of Vinclozolin only, and 45 mg/kg sulforaphane + 100 mg/kg Vinclozolin. The offspring from these crosses were 24.56% NFE2L2$^{+/+}$, 21.05% NFE2L2$^{-/-}$, and 54.39% NFE2L2$^{+/-}$ mice, which were not significantly different based on than the proportions of each genotypic category expected Mendelian ratios in single gene intercrosses between heterozygous parents. To test the hypothesis that the presence of NFE2L2 is required for the rescue effect to occur, we contrast the NFE2L2$^{+/+}$ and NFE2L2$^{-/-}$ for each sulforaphane by vinclozolin.

NFE2L2 is required for sulforaphane to rescue vinclozolin-induced demasculinization. We found that NFE2L2$^{+/+}$ individuals had less severe hypospadias when compared to a NFE2L2$^{-/-}$ individuals (Figure 3A). There was a significant interaction between treatment and genotype (Figure 3D, df=1, χ^2=16.029, LRT p=0.001119) on hypospadias severity such that different genotypes responded to the treatments differently. 45 mg/kg sulforaphane + 100 mg/kg Vinclozolin NFE2L2$^{-/-}$ mice did not display a rescue in hypospadias severity, whereas NFE2L2$^{+/+}$ exposed to 45 mg/kg sulforaphane + 100 mg/kg Vinclozolin did show a morphological rescue. NFE2L2$^{-/-}$ mice had a 2.20 degree of hypospadias severity as measured by M.O.U.S.E., while NFE2L2$^{+/+}$ males reduced the hypospadias severity to 0.89 degree of hypospadias severity, which represented a reduction in severity of 59.55%. The NFE2L2$^{+/+}$, vinclozolin + sulforphane exposed animals displayed low hypospadias severity levels, 0.49. Corn oil control did not show any genotypic differences in hypospadias severity, but conrn oil animals did have a basal level of hypospadias 0.33. They were documented as having hypospadias because they were sampled during development. Interestingly, NFE2L2$^{-/-}$ individuals exposed to sulforaphane did not present with hypospadias, while the NFE2L2$^{+/+}$ were similar to controls (0.50 degree of hypospadias severity). NFE2L2$^{-/-}$ Vinclozolin exposed animals had 1.83 degree of hypospadias severity, while the NFE2L2$^{+/+}$ animals had less severe hypospadias at 1.60.

We found a significant difference between the main effects of treatment and genotype for anogenital distance (Figure 3B, df=1, χ^2=6.1021, LRT p=0.0135), but no significant interaction (LRT p=0.1127). NFE2L2$^{+/+}$ pups had a larger anogenital distance in corn oil (.24%), sulforaphane only (4.28%), and Vinclozolin (2.14%) when compared to similarly treated NFE2L2$^{-/-}$embryos. Anogential distance was 15.63% larger in Vinclozolin + sulforphane exposed NFE2L2$^{+/+}$ mice when compared to NFE2L2$^{-/-}$ mice in the same treatment group.

Discussion

Sulforaphane mitigated the severity of Vinclozolin-induced penis abnormalities and restored masculine development, and this effect was dependent on the transcription factor NFE2L2. We identified

45 mg/kg as the most effective dose to rescue masculine development in mice exposed to 125 mg/kg of Vinclozolin (Figure 1). This effective dose of 45 mg/kg of sulforphane also significantly lowered the potency of Vinclozolin and reduced the dose response relationship between hypospadias incidence, hypospadias severity, PUO height, and septation height, and therefore reduced the effects of antiandrogens on overall urogenital masculinization (Figure 2).

The sulforaphane-induced correction of penis development was not observed in fetal NFE2L2$^{-/-}$ mice exposed to vinclozolin and supplemented with sulforaphane. NFE2L2$^{-/-}$ hypospadias severity and anogenital distance remained at the same severity as Vinclozolin only treated animals, while NFE2L2$^{+/+}$ mice exposed to Vinclozolin and supplemented with sulforaphane displayed a significant rescue in both anogenital distance (increase) and hypospadias severity (decrease) (Figure 3). These results support the hypothesis that the sulforaphane-induced rescue is functioning through the NFE2L2 transcription factor.

The NFE2L2 anti-stress pathway is highly conserved across vertebrates (*213*, *214*), and invertebrate taxa (*215*, *216*) and acts to reduce the effects of both exogenous and endogenous toxins. Several NFE2L2 knockout studies using mice show more severe toxin and stress induced defects when mice have an aberrant NFE2L2 allele (*214*, *217-219*). NFE2L2 functions similarly in humans (*220*). Heavy smokers with certain NFE2L2 alleles were more apt to suffer from low sperm quality, while other smokers with different alleles had normal sperm quality (*221*). DNA sequence variants in the promoter region of human NFE2L2 varied significantly in the transcriptional activity of the gene (*222*) and variants with reduced transcriptional activity show increased risk for human ulcerative colitis (*223*), postmenopausal breast cancer (*224*), and acute lung injury (*222*). Based on the relationship between NFE2L2 activity and the occurrence and severity of hypospadias and other disorders that are linked to exposure to environmental toxicants, we hypothesize sulforaphane may have broad therapeutic effects and that sulforaphane should be evaluated as a prenatal supplement to protect human embryos from a host of environmental pollutants, especially those that are sensitive to NFE2L2 protective activity.

NFE2L2-mediated detoxification

Sulforphane is known to work through a number of mechanisms to ameliorate the effects of toxin exposure and restore normal physiological function (*212, 225, 226*). Here we showed that the sulforaphane reduced anti-androgen-induced hypospadias by acting through NFE2L2. NFE2L2 is a transcription factor that controls the transcription of a number of enzymes and proteins involved in detoxification, including Cytochrome P450s, glutathione S-transferases, gluconurides (*227*). However, NFE2L2 itself requires activation through reduction of disulfide bonds between it and Keap1.

In the absence of sulforphane the adult liver can metabolize Vinclozolin through both Phase I and Phase II detoxification (*228, 229*). Cytochrome P450s (CYP) readily metabolize Vinclozolin into M1 and M2 metabolites, which have stronger antiandrogenic properties than Vinclozolin (*229, 230*). Both M1 and M2 can then become conjugated by Glutathione S-Transferases (GSTs) or UDP-glucuronosyl transferases (UGTs), reducing their anti-androgenic properties, and increasing their excretion from the body (*228*). Sulforaphane-mediated activation of NFE2L2 upregulates CYPs, GSTs, and UGTs within adult livers (*231*), and our study suggests that this upregulation of detoxification enzymes likely occurs within fetal tissues as well. In our study, fetal NFE2L2 was required for the sulforaphane rescue; therefore, we believe NFE2L2 upregulated CYP, GST, and UGT expression in the fetal liver and/or placenta thus reducing circulating levels of Vinclozolin and it metabolites within the fetus. The reduced levels of Vinclozolin and metabolites within the fetus would result in less androgen signaling disruption and in-turn rescued masculine development. Importantly, sulforaphane's mechanism of rescue occurs via a general upregulation of Phase I and Phase II detoxification enzymes and is expected to induce similar types of rescue for different EDCs and different organ systems.

Sulforaphane reduces EDC-induced hypospadias in two strains of mice

Different mouse strains are known to have differential detoxification capacity (*232*). We found that both CD1 and BL6 mice developed hypospadias with vinclozolin exposure and that sulforaphane rescued the vinclozolin-induced hypospadias in both strains. Across the two different strains, therefore, there is a conserved effect of toxicity and toxicity mitigation. Investigation of the ability of sulforaphane

to rescue pollutant-induced hypospadias in other mouse strains such as those in the collaborative cross will help further elucidate the generality of sulforaphane rescue effect congenital defect susceptibility. Indeed, researchers should investigate sulforaphane's ability to correct other pollutant induced defects. NFE2L2 has evolved to facilitate detoxification and reduce oxidative stress, our work suggests that modulating its activity might provide a general protective mechanism against environmental pollutants.

Non-monotonic responses to sulforaphane exposure

We observed a non-monotonic response where the sulforaphane-induced correction peaked at 45 mg/kg and then dropped back to Vinclozolin only levels at 75 and 120 mg/kg of sulforaphane (Figure 1). Non-monotonic responses are typical of NFE2L2-activators (*233*). For example, Hanlon et al. (2008) found that lower doses of sulforaphane were 82% bioavailable for functional use in the mice and was present in blood circulation, while at higher doses the bioavailabity was decreased to 20% and less sulforaphane was found in circulation (*234*). Sulforaphane has a high affinity to bind proteins the authors propose that potential sulforphane binding sites on NFE2L2 were occupied and thus it was metabolized and excreted from the body, thus preventing its mechanism of action (*234*). In our study the reduced effects at higher concentrations might be attributed to a combination of elevated detoxification and clearance, where sulforaphane was metabolized at high rates and cleared from the body before it entered circulation. Maternal removal of sulforaphane would then reduce the concentration that eventually reaches the developing fetus and would prevent pup responses to sulforaphane. This mechanism needs to be formally evaluated, but the removal of excess sulforaphane may be beneficial to prevent over exposure and harmful impacts on the mom and developing fetus.

Reducing hypospadias rates in the human population

Hypospadias is the most common birth defect in the United states and occurs in 1:125 male newborns, on average, each year (*190*). Several Genome Wide Association Studies (GWAS) have failed to find a clear genetic correlate for hypospadias in human patients (*62, 72*). Surely defects in androgen receptors, 5-alpha reductase, and steroidogenic enzymes induce hypospadias, but those mutations only account for a small percentage of hypospadias cases and such mutations cannot propagate through the

population at a rate that can account for the increase in hypospadias occurrence that has occurred over the last few decades. After removing patients with known genetic mutations that induce hypospadias, still 74% of hypospadias patients remained unexplained by genetics (*235*). Within the patients that had hypospadias, but no clear genetic mutations, they had higher toxicant loads (*235*).

Mothers who consume organic fruits and vegetables have a lower probability of having a son with hypospadias, likely due to the reduced pesticide exposure (*236*). Occupational exposures to phthalates (*195*), pesticides (*9*), and other xenoestrogens result in more cases of hypospadias in sons (*195*). GWAS studies show that allelic differences in GSTs explained some proportion of hypospadias cases. GSTs conjugate toxins for secretion and reduced conjugation capacity would result in the reduced capacity to clear toxicants from the body (*156*). These data suggest that exposure to environmental EDCs are responsible for many cases of hypospadias with susceptibilities related to lifestyle, occupation, and genetics. Detoxification efficiency may determine how responsive an individual is to contaminant exposure and may be the deciding factor concerning normal vs abnormal development. Sulforaphane is known to increase detoxification efficiency and could help buffer embryos from harmful pollutants by stimulating the transcription of genes that encode enzymes and proteins that are involved in detoxification of chemicals that induce aberrant development and toxicant-related disease processes

These findings reported here indicate that the incidence of the congenital defect hypospadias and its severity can be reduced by activating NFE2L2 through prenatal supplementation of sulforaphane. Previous studies have shown that NFE2L2 can mitigate a number of toxin-induced diseases through the activation of transcription of genes that engage in detoxification or have antioxidant activities (*213, 214, 218, 219, 226*). In the context of hypospadias more research must be conducted on the underlying mechanism through which this rescue effect occurs. Future studies should investigate other supplements that may mitigate hypospadias severity and if rescue can happen with complex mixture and more environmentally relevant exposure. This study combined with past sulforaphane studies provide

important steps toward the development of targeted prenatal therapeutics to reduce environmental toxicant induced birth defects such as hypospadias.

Material and Methods

Mouse maintenance

All studies were carried out under an approved protocol established by East Carolina University (ECU) Institutional Animal Care and Use Committee (AUP D-297a). Eight-week-old CD-1 mice were purchased (Charles River Breeding Laboratories Raleigh, NC) and acclimated for at least 7 days to 70-72 F on a 12 h light-dark cycle with free access to food and water (Purina ISOCHOW). CD1 mice were time mated by setting 1-2 female mice with a male mouse. Vaginal plugs were checked each morning during the hours of 8:00-10:00 am. The presence of a vaginal plug indicated mating had occurred and the dam was recorded as being embryonic day (E) 0.5 at noon that day. Pregnant dams were removed from the male's cage and were housed with same treatment dams or singingly.

Eight-week-old C57BL/6J female mice and eight-week-old B6.129X1-Nfe2l2^{tm1Ymk}/J (NFE2L2 knockout) mice were purchased (The Jackson Laboratory, Bar Harbor, ME) and acclimated using the same method as that which was used for the CD-1 mice. Wildtype females and knockout males were mated to generate a colony of NFE2L2 heterozygous mice. Non-sibling NFE2L2 heterozygous mice were then mated as indicated above to generate pups that were on average 25% wildtype, 25% knockout, and 50% heterozygous.

Chemical preparation

Vinclozolin (Sigma Aldrich, St. Louis, MO, United States,45705), a model anti-androgenic endocrine disrupting pesticide, and its metabolites are known to competitively inhibit the ability of androgen to bind to the androgen receptor and have been used to consistently induce hypospadias in previous studies (*176, 177, 237*). Vinclozolin doses 10, mg, 15mg, 20mg, or 25mg of Vinclozolin were dissolved in 0.45mL Tocopherol stripped corn oil (Millipore, Santa Ana, CA, United States, 02901415) for doses 50, 75, 100, and 125 mg/kg respectively. The mixture was heated at 60 C and mixed with

Genemate Rotator (H-6800) for 3 hours. Dose volume (μL) received by the pregnant dam was 2.5 multiplied by the weight of the pregnant dam.

Sulforaphane is derived from broccoli (12), and has been used in several mouse studies to induce detoxification and reduce the effects of toxicants. Once Vinclozolin was dissolved in corn oil, sulforaphane was removed from -20 C storage and either 0mg, 1.5mg, 3mg, 6mg, 9mg, 12mg, 15mg, or 24mg was combined with corn oil. The corn oil sulforaphane mixture was centrifuged for five seconds and the mixture was pipetted into the dissolved Vinclozolin solution. The Vinclozolin + sulforaphane + corn oil mixture was then vortexed for one minute and was visually inspected to ensure that the Vinclozolin did not fall out of solution and the sulforaphane was completely in solution. Vinclozolin never fell out of solution, but if sulforphane was not completely dissolved (cloudiness to the corn oil), the mixture was vortexed for another 30 seconds and reevaluated. In all cases sulforaphane went into solution within 1 minute of mixing.

Sulforaphane dose response with a single Vinclozolin dose

To test if sulforaphane exposure can mitigate Vinclozolin-induced malformations, pregnant CD-1 dams were gavaged from embryonic day (E) 13.5-E16.5 with 125 mg/kg Vinclozolin and one of eight doses of sulforaphane (0, 7.5, 15, 30, 45, 60, 75, and 120 mg/kg (n_{dam}=3-5)). Twenty-four hours after the last dose on E18.5, the dams were humanely sacrificed. Embryos were individually weighed, and genitalia were evaluated for hypospadias severity using methods adapted from the published, standardized scoring system (M.O.U.S.E) (7).

Vinclozolin dose response with a single sulforaphane dose

To test if sulforaphane reduced the potency of the antiandrogen Vinclozolin by reducing its dose response relationship, pregnant CD-1 dams were gavaged from embryonic day (E) 13.5-E16.5 with either 0 mg/kg or 45mg/kg of sulforaphane across Vinclozolin one of five doses (0, 50, 75, 100, and 125 mg/kg

(n_{dam}=3-6)). On E18.5, the dams were humanely sacrificed. Embryos were individually weighed, and genitalia were evaluated for hypospadias severity using the standardized scoring system (M.O.U.S.E) (7).

NFE2L2 knockout study.

To test whether activation of the NFE2L2 transcription factor is the molecular mechanism through which sulforaphane is acting we conducted a NFE2L2 knockout study. Pregnant dams were exposed to either corn oil (control), 100 mg/kg of vinclozolin, 45 mg/kg of sulforaphane, or 45 mg/kg sulforaphane + 100 mg/kg of vinclozolin on E13.5-16.5 and were sacrifice on E18.5. Vinclozolin dose (100 mg/kg) was selected for this experiment because it was the lowest vinclozolin dose that induced 100% hypospadias in the vinclozolin dose response study (above). Pup length, weight, and anogenital distance were measured at dissection. The embryos were sexed based on the presence of testis or ovaries and external genitalia were stored in 10% neutral buffered formalin for morphological analysis. Tail snips from each pup were frozen in liquid nitrogen for later genotyping. Hypospadias severity was compared between the NFE2L2 knockout (-/-) and wild-type (+/+) across the different treatments.

Morphological data collection

External genitalia were histologically analyzed for hypospadias as previously described in (*238*). Briefly genitalia were serially sectioned perpendicular to the proximal to distal axis of the penis (10 μm) and then stained with hematoxylin (Newcomer Supply, Middleton, WI, United States, 1201) and eosin (Newcomer Supply, Middleton, WI, United States, 1072) and microscopically evaluated. Proximal urethral opening height and sepation height data were collected. A combination of 100x and 200x objectives on the Leica DME microscope was used to identify and define relevant histological landmarks.

DNA extraction and PCR

DNA extraction was conducted on 2mm-6mm subsamples from fetal tail samples. Tail snips were homogenized in .6mL of Cetrimethyl ammonium bromide (CTAB) buffer (Teknova, C2190, Hollister, CA). Homogenate was mixed with 3.6μL of proteinase K (Invitrogen, 25530049, Carlsbad, CA) and incubated at 65 C for 2-3 hours. After incubation 600μL of chloroform (VWR, 0757, Randor, PA) as

added to each tube and centrifuged at 10,000 rpm for 10 minutes. The top layer of the centrifuged sample was transferred to a new tube and 1mL of cold 100% molecular-grade ethanol (Pharmaco-AAPER, 111000200, Brookfield, CT) was added to each tube. Sample were again centrifuged at 10,000 rpm for 10 minutes. The mixture was dumped out, preserving the pellet at the bottom of the tube and 1mL of cold 70% ethanol was added to each tube and centrifuged at 10,000 rpm for 10 minutes. The ethanol was removed from the tube and another 1mL of cold 70% ethanol was added to the pellet and centrifuged at 10,000 rpm for 10 minutes. The last wash of ethanol was dumped from the tube and open tubes were placed in the 60 C heat block until all ethanol had evaporated (~5 min). 50µL of molecular grade water (Quality Biological, 351-029-101, Gaithersburg, MD) was added to the tubes, centrifuged at 10,000 rpm for 2 minutes and place in 4 C overnight.

DNA quantity and quality were measured with the nanodrop (Thermoscientific nanodrop 2000) and documented. A260/A280 values >1.7 were deemed high enough quality for DNA analysis. For PCR, 100ng of DNA was added to each .2mL PCR tube. Then µL volume of 100ng DNA - 8µL of water was added to the tube to bring reaction to a total volume of 25µL along with 12.5µL of PCR mastermix (Promega, M750B, Madison, WI), 1.5µL common primer (GCCTGAGAGCTGTAGGCCC), 1.5µL wildtype reverse primer (GGAATGGAAAATAGCTCCTFCC), and 1.5µL mutant reverse primer (GACAGTATCGGCCTCAGGAA) (Invitrogen, Carlsbad, CA). Primer sequences designed for the NFE2L2 knockout mouse were obtained from Jackson Laboratory (*https://www2.jax.org/protocolsdb/f?p=116:5:0::NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS_CODE:26266,017009)*. Reactions were put in the thermocycler for 1) 2 minutes at 94 C, 2) then 94 C for 20 seconds, 3) 65 C for 15 seconds, and 4) 68 C for 10 seconds. Steps 2-4 were repeated 10 times and step 3 decrease 1.5 C each cycle. Then the sample went through 5) 94 C for 15 seconds, 6) 50 C for 15 seconds, and 7) 72 C for 10 seconds. Steps 5-7 were repeated 28 times and then the samples sat at 72 C for 2 minutes and then 10 C until they were transferred to the gel. While the PCR reaction was running 4.5g of agarose (Invitrogen, 16500-100, Carlsbad, CA) was added to 150mL of 1X TAE (Fisher, BP1330-1,

Waltham, MA) to make a 3% agarose gel. Ethidium bromide (1.5μL/50mL of gel) (VWR, X328, Randor, PA) was added to the gel to visualize DNA. PCR products were mixed with 2μL of dye (VWR, 1B1812, Randor, PA)and then the entire reaction was added to the gel. A ladder (VWR, E854-100rxn, , Randor, PA) was added to the first and last wells of each gel. The gel was run at 96 volts for ~45 minutes, or until pink bands reached the bottom of the gel. The gel was then imaged with (Biorad) and bands were assessed and pups were then classified as wildtype, heterozygote, or knockout depending on band presence. DNA length for wildtype and mutant alleles were 262bp and 400bp respectively. Heterozygotes were determined when both bands were present.

Statistical analysis

All data were analyzed in R statistical programming environment v. 3.4.3 (*180*) (Supplemental R code). All analyses were conducted with the lme4 package (*163*). Assumptions of homoscedasticity, normality, and dispersion were tested by interpreting residual plots, qqplots, and dispersion coefficients, respectively.

Hypospadias incidence was analyzed using a mixed model binomial regression. PUO height and urorectal septation height data were analyzed as raw counts of 10 μm histological sections and a regression was performed using a Poisson distribution. A Poisson error distribution was used to phenomenologically describe the relationships between dose, exposure window and the response (section counts). Penis length was used to allometrically correct for urethral length as previously described(*129*).

Hypospadias severity score (MOUSE) proportionally scales with urethral length determined histologically (*129*) and is on an interval scale. Mean hypospadias severity was analyzed with a normal error distribution using linear mixed-models. To avoid pseudoreplication, dam was treated as a random effect in all models with pups being nested within dam (*181*). Nested models were compared with likelihood ratio tests (LRT) using $p<0.05$ to establish the best model fit (*181*).

Acknowledgements
We would like to thank the Mike McCoy lab for critical feedback on experimental design and statistical analysis, the ECU veterinary staff for caring for the animals, Ariane Peralta for opening her lab space to use, and April Blakeslee and Rebecca Barnard for training us on DNA extraction and PCR techniques. We also thank Jacques Robidoux for helpful feedback and suggesting sulforaphane as a nutritive supplement.

Figures

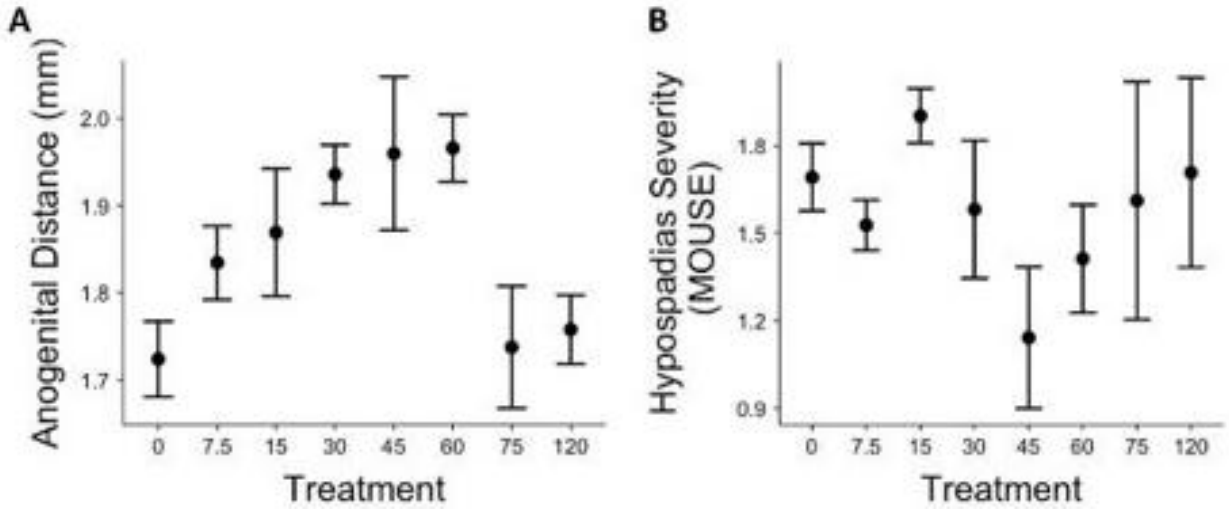


Figure 5. 1 Sulforaphane rescues Vinclozolin-induced hypospadias and anogenital distance. **A and B)** Mice exposed to 125 mg/kg of Vinclozolin and a dose response of sulforaphane displayed a non-monotonic restoration of masculinization. **A)** Anogenital distance showed the largest restoration at 45 mg/kg of sulforaphane and diminished rescue at 75 and 120 mg/kg. **B)** Hypospadias severity did not show the same non-monotonic response, but showed the largest rescue of severity at 45 mg/kg of sulforaphane. Error bar represent standard error. * indicate $p<0.05$ when treatment was compared to 0 mg/kg of sulforaphane.

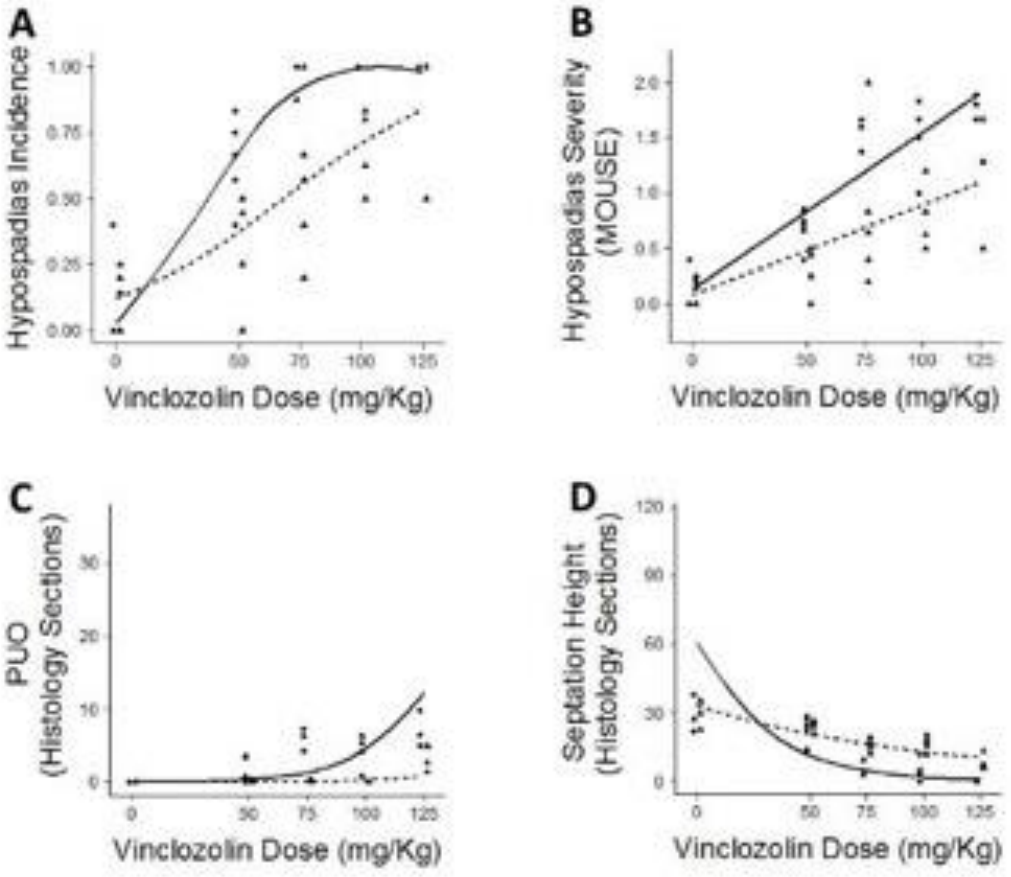


Figure 5. 2 Sulforaphane's rescue occurs across a Vinclozolin dose response.

A and B) Hypospadias incidence and severity were significantly rescued with 45 mg/kg of sulforaphane (dashed) supplementation when compared to Vinclozolin only treatments (solid). Both incidence and severity showed difference in the mean from 50 – 125 mg/kg of Vinclozolin exposure. **C and D)** Sulforphane exposure also reduced the size of proximal urethral openings and increased the height of septation the penis.

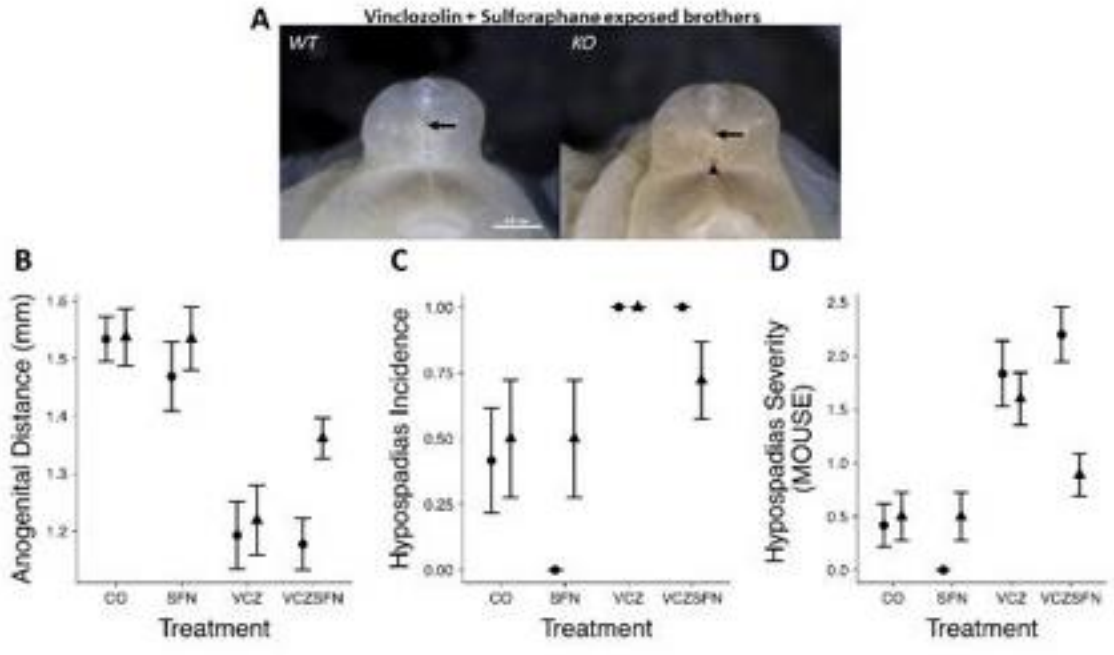


Figure 5. 3 NFE2L2 is driving the sulforaphane-mediated rescue of hypospadias.

A) Penises from NFE2L2$^{+/+}$ and NFE2L2$^{-/-}$ brothers show that NFE2L2$^{+/+}$ has less severe hypospadias (black arrow) and lacks a proximal urethral opening (black arrowhead). **B)** Anogenital distance did not differ between corn oil control and sulforaphane only mice or genotypes. Vinclozolin exposure induced a significant decrease in anogenital distance in both NFE2L2$^{+/+}$ and NFE2L2$^{-/-}$ mice and Vinclozolin + sulforaphane restore masculine anogenital distance in NFE2L2$^{+/+}$, but not NFE2L2$^{-/-}$ mice. **C)** Hypospadias incidence was not significant different between corn oil or sulforaphane treatments or genotypes. Vinclozolin induced 100% of hypospadias in mice and Vinclozolin + sulforaphane reduced hypospadias incidence in NFE2L2$^{+/+}$ mice (to what %), but not NFE2L2$^{-/-}$. **D)** Hypospadias severity displayed a similar response to B and C where no differences were observed in corn oil or sulforaphane treated animals, while Vinclozolin significantly increase hypospadias severity. With Vinclozolin + sulforaphane treatments NFE2L2$^{+/+}$ mice had reduced hypospadias severity and NFE2L2$^{-/-}$ did not.

Conclusions

Hypospadias is the second most common birth defect in the United States (*6*). Endocrine disrupting chemicals (EDCs) induce several hypospadias cases throughout the world (*105*, *112*), however, there is still much to be learned about normal penis development and how EDCs perturb that development to induce birth defects such as hypospadias.

In this dissertation, I reviewed the current knowledge of external genitalia development, and discuss that the balance of sex hormones within the developing fetus is important for correct genitalia formation in both sexes. I also highlight the bias in the literature to study the role of androgens in males rather than the mechanisms through which sex hormones affected genitalia development generally. With geometric morphometrics and the anti-androgen, vinclozolin, I studied normal sexual dimorphism and the impact of an anti-androgenic EDC on sexually dimorphic genitalia development. Morphological sexual dimorphism and vinclozolin induced feminization of the male external genitalia occurred on E15.5, further suggesting the anti-androgenic nature of vinclozolin during male development. Therefore, investigating the molecular mechanisms and developmental processes prior to E15.5 is essential for understanding the origins of feminization of the genitalia and development of hypospadias. Vinclozolin's feminization of male development oscillated across time where E15.5 was more feminized than E16.5 and E17.5 was more feminized than E16.5 and E18.5. This pulsatile response to vinclozolin shows that development of the male external genitalia must include mechanisms and processes that attempt to restore masculine development in response to altered endocrine signaling and exposure to EDCs. The endocrine system functions via positive and negative feedback networks that ensure proper development (*1*). The male fetus might increase

testosterone production, reduce aromatization of testosterone to estrogen, or increase vinclozolin detoxification enzymes in effort to restore appropriate endocrine signaling, but at high vinclozolin concentrations still develops abnormally.

In females, vinclozolin exposure masculinized the genitalia showing that the response to vinclozolin and possibly other EDCs is sex specific and much more complex than originally considered. The masculinization of the female genitalia suggests that the lack of androgen signaling plays a role in female development. Alternatively, the masculinization of the female external genitalia could be driven by the increased removal of circulating estrogens by detoxifying enzymes in the liver. Vinclozolin is known to activate the expression of many phase I and phase II detoxification enzymes (data not shown) in the liver which may lead to the aberrant removal of circulating estrogen. Reduced estrogen signaling in the female external genitalia is known to induce more masculine phenotypes. The idea that there is a balance of estrogen and testosterone signaling that is required during development to generate sexually dimorphic genitalia further exemplifies the importance of appropriate sex steroid signaling during development. Alternatively, vinclozolin could be functioning as an androgen agonist, in females, as it does bind the androgen receptor. Wong et al. 1998 (*239*) showed that in cell culture vinclozolin reduced androgen receptor activation in cultures with androgen present. In androgen deficient cultures, however, vinclozolin exposure resulted in elevated androgen signaling. Females have little circulating testosterone so vinclozolin may be activating androgen receptor in the female external genitalia.

Next, I focused specifically on determining the mechanisms inducing differences in the severity of hypospadias and developed a standardized method for quantifying disrupted morphogenesis. First, I developed a standardized scoring system that allows the quantification of

hypospadias severity called the Mouse Objective Urethral Evaluation (MOUSE). This system was inspired by the HOPE score (*109*) used in humans to evaluate hypospadias in humans and was based on previous studies that identified large morphological changes that occur in the preputial swellings and urethral exit of them mouse (*49, 50, 119, 175*) external genitalia. MOUSE outlines several aspects of genitalia morphology that are critical for correct classification into separate categories in a dichotomous key and provides a training protocol that ensures the observer is providing precise and accurate scores. The dichotomous key allows for hypospadias severity to be scored on a scale of 0-3 that based on how similar penis morphology was to a normal male (0) or normal female (3) genital morphology. This standardized approach allows for a quantitative analysis of hypospadias severity. MOUSE allows for a universal hypospadias severity quantification analyses, and comparisons across studies and lab groups will be easier and aid in ascertaining the toxicants and genes involved in aberrant penis development. In fact, a recent study has used and adapted MOUSE to investigate how diethylstilbesterol, a potent estrogen, influences the severity of hypospadias in both male and female external genitalia (*240*). I used MOUSE to ask questions about vinclozolin's impact on sensitive time periods in development.

Using MOUSE and other histological measurements I found that androgen receptor positively feeds back to increase its own expression through development. Vinclozolin exposure disrupts this feed forward loop and induces more severe penis abnormalities with earlier vinclozolin exposure. When mice were exposed to the anti-androgen vinclozolin on E13.5 and androgen receptor was compared to non-exposed embryos on E14.5 there was a reduction in androgen receptor expression in the external genitalia. This finding suggested that early vinclozolin exposure disrupts a feed forward loop and induces more severe penis abnormalities

relative to later exposure. I tested whether this reduction in AR was important for correct penis development and showed that mice exposed on E13.5-16.5 had more severe hypospadias and other penile abnormalities relative to those exposed only on E14.5-16.5. This finding was true even after correcting for total vinclozolin dose. Early reduction of androgen receptor sensitizes the penis to further anti-androgen perturbations and leads to more severe hypospadias cases. Variation in both timing and dose of exposure in humans likely influences the child's susceptibility to developing hypospadias. Human hypospadias incidence and severity are strongly correlated with the concentration of contaminants in fetal tissue and is associated with the season of conception (*100, 236, 241*).

After conducting research that allowed me to effectively increase hypospadias severity with differential dosing regimens I was poised to ask if I could reduce hypospadias severity by providing the vinclozolin-exposed pregnant mouse dams with a known nutraceutical. Sulforaphane is a derivative of broccoli and is known to increase the expression of detoxification and antioxidant enzymes throughout the body (*205*). Sulforaphane was a strong candidate with high potential to reduce the effects of EDCs because its ability to increase detoxification was expected to reduce the internal dose of the EDC, vinclozolin, in exposed mice. I found that sulforaphane restored masculine development even at very high doses of vinclozolin and that sulforaphane can reduce the dose response curve of vinclozolin. Sulforaphane exposure resulted in less severe hypospadias and other penis abnormalities. The restoration induced by sulforaphane was effective up to 60 mg/kg, while 75 and 120 mg/kg of sulforaphane resulted in a loss of developmental rescue. These very high doses of sulforaphane may result in auto-detoxification, which would prevent sulforaphane from carrying out its beneficial action. Also,

the excessive induction of antioxidants can change the redox state of the cells and result in aberrant apoptosis or cell proliferation (*242*).

Throughout the literature sulforaphane is hypothesized to induce detoxification and antioxidant enzyme through activation of Nrf-2 (*207*, *212*). To test if Nrf-2 was required for the sulforaphane induced rescue I used Nrf-2 knockout mice. Indeed, the sulforaphane rescue required Nrf-2 to restore normal masculine development. Sulforaphane's rescue is likely occurring by inducing detoxification enzymes within the dam and fetus, which then results in removal of vinclozolin from the body and thus less vinclozolin-induced effects on male urogenital development. The NFE2L2 allelic changes were in the pup genome, so I suspect the fetal tissue is largely contributing to the sulforaphane-induced rescue. Both the liver and placenta are strong candidates for detoxification sites in the fetus. Alternatively, the NFE2L2-dependent antioxidant enzyme upregulation could reduce oxidative stress in several tissues. Vinclozolin was identified to induce aberrant reactive oxygen species (ROS) in liver microsomes (*243*). Elevated ROS in either the fetal testis or penis could prove detrimental. Elevated ROS in the testis results in diminished testosterone production and thus result in less androgen signaling at the penis (*244*). Aberrant ROS in the penis could elevate the amount of protein carbonyl adducts on developmental proteins and nuclear receptors. The accumulated adducts disrupt normal protein function and can derail normal developmental signaling. Sulforaphane induction of antioxidant enzymes could prevent the deleterious impacts of ROS on the testis and penis.

In conclusion, I have developed standardized methods to measure external genitalia development, found androgen receptor positive feedback is essential for normal penis development, and found that EDC-induced hypospadias severity can be reduced with the NFE2L2-activator, sulforaphane. This work facilitated our understanding of the drivers of

hypospadias severity and takes a critical first step toward developing a prenatal preventative therapy for one of the most common birth defects in the world, hypospadias.

References

1. H. Kitano, Biological robustness. *Nature reviews. Genetics* 5, 826 (Nov, 2004).
2. J. F. Couse, M. M. Yates, V. R. Walker, K. S. Korach, Characterization of the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) Null mice reveals hypergonadism and endocrine sex reversal in females lacking ERalpha but not ERbeta. *Molecular endocrinology* 17, 1039 (Jun, 2003).
3. E. L. Aschim *et al.*, Linkage between cryptorchidism, hypospadias, and GGN repeat length in the androgen receptor gene. *The Journal of clinical endocrinology and metabolism* 89, 5105 (Oct, 2004).
4. E. Mylchresst, D. G. Wallace, R. C. Cattley, P. M. D. Foster, Dose-Dependent alterations in androgen-regulated male reproductive development in rats exposed to Di(n-butyle) phthalate during late gestation. *Toxicol Sci* 55, 143 (2000).
5. C. P. Nelson *et al.*, THE INCREASING INCIDENCE OF CONGENITAL PENILE ANOMALIES IN THE UNITED STATES. *The Journal of Urology* 174, 1573 (10//, 2005).
6. L. J. Paulozzi, J. D. Erickson, R. J. Jackson, Hypospadias Trends in Two US Surveillance Systems. *Pediatrics* 100, 831 (1997).
7. M. J. Cohn, Development of the external genitalia: conserved and divergent mechanisms of appendage patterning. *Developmental dynamics : an official publication of the American Association of Anatomists* 240, 1108 (May, 2011).
8. G. Yamada *et al.*, Molecular genetic cascades for external genitalia formation: an emerging organogenesis program. *Developmental dynamics : an official publication of the American Association of Anatomists* 235, 1738 (Jul, 2006).
9. C. M. Rocheleau, P. A. Romitti, L. K. Dennis, Pesticides and hypospadias: a meta-analysis. *Journal of pediatric urology* 5, 17 (Feb, 2009).
10. K. P. Stillerman, D. R. Mattison, L. C. Giudice, T. J. Woodruff, Environmental exposures and adverse pregnancy outcomes: a review of the science. *Reproductive sciences* 15, 631 (Sep, 2008).
11. P. J. Hynes, J. P. Fraher, The development of the male genitourinary system. I. The origin of the urorectal septum and the formation of the perineum. *Br J Plast Surg* 57, 27 (2004).
12. P. J. Hynes, J. P. Fraher, The development of the male genitourinary system: II. The origin and formation of the urethral plate. *Br J Plast Surg* 57, 112 (Mar, 2004).
13. A. W. Seifert, B. D. Harfe, M. J. Cohn, Cell lineage analysis demonstrates an endodermal origin of the distal urethra and perineum. *Developmental biology* 318, 143 (Jun 01, 2008).
14. A. Dean, R. M. Sharpe, Clinical review: Anogenital distance or digit length ratio as measures of fetal androgen exposure: relationship to male reproductive development and its disorders. *The Journal of clinical endocrinology and metabolism* 98, 2230 (Jun, 2013).
15. A. Thankamony, V. Pasterski, K. K. Ong, C. L. Acerini, I. A. Hughes, Anogenital distance as a marker of androgen exposure in humans. *Andrology* 4, 616 (Jul, 2016).
16. M. H. Hsieh, B. N. Breyer, M. L. Eisenberg, L. S. Baskin, Associations Among Hypospadias, Cryptorchidism, Anogenital Distance, and Endocrine Disruption. *Journal of pediatric urology* 9, 137 (2008).
17. U. Drews, Helper function of the Wolffian ducts and role of androgens in the development of the vagina. *Sex Dev* 1, 100 (2007).
18. C. M. Amato, M. Boyd, J. Yang, K. McCoy, Early anti-androgen exposure potentiates the effects of continued exposure on penis development. *Biol Reprod*, (2018).
19. A. Murashima, S. Kishigami, A. Thomson, G. Yamada, Androgens and mammalian male reproductive tract development. *Biochimica et biophysica acta* 1849, 163 (Feb, 2015).

20. **C. W. Bardin, J. F. Catterall, Testosterone: A Major Determinant of Extragenital Sexual Dimorphism. *Science* 211, 1285 (1981).**
21. **C. K. Matson *et al.*, DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature* 476, 101 (Jul 20, 2011).**
22. **N. H. Uhlenhaut *et al.*, Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell* 139, 1130 (Dec 11, 2009).**
23. **P. J. O'Shaughnessy, P. J. Baker, M. Heikkila, S. Vainio, M. A. P., Localization of 17B-Hydroxysteroid Dehydrogenase /17-Ketosteroid Reductase Isoform Expression in the Developing Mouse Testis- Androstenedione is the Major Androgen Secreted by Fetal/Neonatal Leydig Cells. *Endocrinology* 141, 2631 (2000).**
24. **Y. Shima *et al.*, Contribution of Leydig and Sertoli cells to testosterone production in mouse fetal testes. *Molecular endocrinology* 27, 63 (Jan, 2013).**
25. **Z. Zheng, B. A. Armfield, M. J. Cohn, Timing of androgen receptor disruption and estrogen exposure underlies a spectrum of congenital penile anomalies. *Proceedings of the National Academy of Sciences of the United States of America* 112, E7194 (Dec 29, 2015).**
26. **H. Suzuki, S. Matsushita, K. Suzuki, G. Yamada, 5alpha-Dihydrotestosterone negatively regulates cell proliferation of the periurethral ventral mesenchyme during urethral tube formation in the murine male genital tubercle. *Andrology* 5, 146 (Jan, 2017).**
27. **H. Tian, D. W. Russell, Expression and Regulation of Steroid 5 alpha Reductase in the Genital Tubercle of the Fetal Rat. *Developmental Dynamics* 209, 117 (1997).**
28. **M. Ranjan *et al.*, Comparative study of human steroid 5a-reductase isoforms in prostate and female breast skin tissues: sensitivity to inhibition by finasteride and epristeride. *Life Sciences* 71, 115 (2002).**
29. **M. Welsh, D. J. MacLeod, M. Walker, L. B. Smith, R. M. Sharpe, Critical androgen-sensitive periods of rat penis and clitoris development. *International journal of andrology* 33, e144 (Feb, 2010).**
30. **A. K. Hotchkiss *et al.*, Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. *Toxicol Sci* 96, 335 (Apr, 2007).**
31. **C. J. Wolf, A. Hotchkiss, J. S. Ostby, G. A. LeBlanc, L. E. G. Jr.*, Effects of Prenatal Testosterone Propionate on the Sexual Development of Male and Female Rats: A Dose-Response Study. *Toxicol Sci* 65, 71 (2002).**
32. **D. El-Maouche, W. Arlt, D. P. Merke, Congenital adrenal hyperplasia. *The Lancet*, (2017).**
33. **P. W. Speiser *et al.*, Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an Endocrine Society clinical practice guideline. *The Journal of clinical endocrinology and metabolism* 95, 4133 (Sep, 2010).**
34. **P. C. WHITE, P. W. SPEISER, Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency. *Endocr Rev* 21, 245 (2000).**
35. **P. W. Speiser *et al.*, Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J Clin Invest* 90, 584 (Aug, 1992).**
36. **F. M. Bentvelsen *et al.*, Developmental pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat. *Molecular and Cellular Endocrinology* 113, 245 (1995).**
37. **K. KUBOTA *et al.*, Effects of vinclozolin administration on sperm production and testosterone biosynthetic pathway in adult male rat. *Journal of Reproduction and Development* 40, 403 (2003).**

38. D. A. Wolf, T. Herzinger, H. Hermeking, D. Blaschke, W. Horz, Transcriptional and Posttranscriptional Regulation of Human Androgen Receptor Expression by Androgen *Molecular endocrinology* **7**, 924 (1993).
39. E. Mylchreest, M. Sar, D. G. Wallace, P. M. D. Foster, Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(n-butyl) phthalate. *Reprod Toxicol* **16**, 19 (2002).
40. K. J. Turner *et al.*, Altered gene expression during rat Wolffian duct development in response to in utero exposure to the antiandrogen linuron. *Toxicol Sci* **74**, 114 (Jul, 2003).
41. A. CM, B. M, Y. J, M. KM, Early anti-androgen exposure potentiates the effects of continued exposure on mouse penis development. (2018).
42. S. Yoshimura *et al.*, Hypospadias and incomplete preputial separation in male rats induced by prenatal exposure to an anti-androgen, Flutamide. *J Toxicol Pathol* **17**, 113 (2004).
43. M. Welsh *et al.*, Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest* **118**, 1479 (Apr, 2008).
44. C. J. Wolf, G. A. LeBlanc, J. S. Ostby, L. E. G. Jr, Characterization of the period of sensitivity of fetal male sexual development to Vinclozolin. *Toxicol Sci* **55**, 152 (2000).
45. M. L. Vilela *et al.*, Endocrine disruptors and hypospadias: role of genistein and the fungicide vinclozolin. *Urology* **70**, 618 (Sep, 2007).
46. D. W. Silversides, C. A. Price, G. M. Cooke, Effects of short-term exposure to hydroxyflutamide in utero on the development of the reproductive tract in male mice. *Can. J. Physiol. Pharmacol* **73**, 1582 (1995).
47. R. L. Clark *et al.*, External genitalia abnormalities in male rats exposed in-utero to Finasteride, a 5alpha-reductase inhibitor. *Teratology* **42**, 91 (1990).
48. S. Miyagawa *et al.*, Genetic interactions of the androgen and Wnt/beta-catenin pathways for the masculinization of external genitalia. *Molecular endocrinology* **23**, 871 (Jun, 2009).
49. K. Suzuki *et al.*, Sexually dimorphic expression of Mafb regulates masculinization of the embryonic urethral formation. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 16407 (Nov 18, 2014).
50. A. Petiot, C. L. Perriton, C. Dickson, M. J. Cohn, Development of the mammalian urethra is controlled by Fgfr2-IIIb. *Development* **132**, 2441 (May, 2005).
51. J. Cheung-Flynn *et al.*, Physiological role for the cochaperone FKBP52 in androgen receptor signaling. *Molecular endocrinology* **19**, 1654 (Jun, 2005).
52. H. Chen *et al.*, Fkbp52 regulates androgen receptor transactivation activity and male urethra morphogenesis. *The Journal of biological chemistry* **285**, 27776 (Sep 03, 2010).
53. H. Nishida *et al.*, Gene expression analyses on embryonic external genitalia: identification of regulatory genes possibly involved in masculinization processes. *Congenital anomalies* **48**, 63 (Jun, 2008).
54. C. L. HAYES *et al.*, 17B-Estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 9776 (1996).
55. A. J. Lee, M. X. Cai, P. E. Thomas, A. H. Conney, B. T. Zhu, Characterization of the oxidative metabolites of 17beta-estradiol and estrone formed by 15 selectively expressed human cytochrome p450 isoforms. *Endocrinology* **144**, 3382 (Aug, 2003).
56. A. K. Pingili *et al.*, 6beta-hydroxytestosterone, a cytochrome P450 1B1 metabolite of testosterone, contributes to angiotensin II-induced hypertension and its pathogenesis in male mice. *Hypertension* **65**, 1279 (Jun, 2015).

57. **A. W. Seifert, C. M. Bouldin, K. S. Choi, B. D. Harfe, M. J. Cohn, Multiphasic and tissue-specific roles of sonic hedgehog in cloacal septation and external genitalia development. *Development* 136, 3949 (Dec, 2009).**

58. **M. Harada *et al.*, Tissue-specific roles of FGF signaling in external genitalaia development *Developmental Dynamics* 244, 759 (2015).**

59. **J. A., Recherches sur la différenciation sexuelle de l'embryon de lapin. III. Rôle des gonades foetales dans la différenciation sexuelle so- matique. *Arch Anat Microsc Morph* Exp 36, 271 (1947).**

60. **L. A. Ipulan *et al.*, Development of the external genitalia and their sexual dimorphic regulation in mice. *Sex Dev* 8, 297 (2014).**

61. **M. M. Dominguez, R. M. Liptrap, P. K. Basrur, Steroidogenesis in Fetal Bovine Gonads. *Can J Vet Res* 52, 401 (1988).**

62. **Z. Wang *et al.*, Up-regulation of estrogen responsive genes in hypospadias: microarray analysis. *J Urol* 177, 1939 (May, 2007).**

63. **S. Yucel, A. G. Cavalcanti, D. Z. Wang, L. S. Baskin, The effect of oestrogen and testosterone on the urethral seam of the developing male mouse genital tubercle. *BJU Internation* 92, 1016 (2003).**

64. **K. Agras, E. Willingham, Y. Shiroyanagi, P. Minasi, L. S. Baskin, Estrogen Receptor-α and β are Differentially Distributed, Expressed and Activated in the Fetal Genital Tubercle. *The Journal of Urology* 177, 2386 (2007).**

65. **J. H. Yang, J. Menshenina, G. R. Cunha, N. Place, L. S. Baskin, Morphology of mouse external genitalia: implications for a role of estrogen in sexual dimorphism of the mouse genital tubercle. *J Urol* 184, 1604 (Oct, 2010).**

66. **I. Paterni, C. Granchi, J. A. Katzenellenbogen, F. Minutolo, Estrogen receptors alpha (ERalpha) and beta (ERbeta): subtype-selective ligands and clinical potential. *Steroids* 90, 13 (Nov, 2014).**

67. **M. Bondesson, R. Hao, C. Y. Lin, C. Williams, J. A. Gustafsson, Estrogen receptor signaling during vertebrate development. *Biochimica et biophysica acta* 1849, 142 (Feb, 2015).**

68. **T. H. Charn *et al.*, Genome-wide dynamics of chromatin binding of estrogen receptors alpha and beta: mutual restriction and competitive site selection. *Molecular endocrinology* 24, 47 (Jan, 2010).**

69. **K. Suk Kim *et al.*, Induction of hypospadias in a murine model by maternal exposure to synthetic estrogens. *Environmental Research* 94, 267 (2004).**

70. **S. Jesmin *et al.*, Aromatase is abundantly expressed by neonatal rat penis but downregulated in adulthood. *Journal of molecular endocrinology* 33, 343 (Oct, 2004).**

71. **B. Liu *et al.*, Activating transcription factor 3 is up-regulated in patients with hypospadias. *Pediatric research* 58, 1280 (Dec, 2005).**

72. **N. Kalfa *et al.*, Genomic Variants of ATF3 in Patients With Hypospadias. *The Journal of Urology* 180, 2183 (2008).**

73. **H. Wang *et al.*, The stress response mediator ATF3 represses androgen signaling by binding the androgen receptor. *Molecular and cellular biology* 32, 3190 (Aug, 2012).**

74. **B. Liu, K. Agras, E. Willingham, M. L. Vilela, L. S. Baskin, Activating transcription factor 3 is estrogen-responsive in utero and upregulated during sexual differentiation. *Hormone research* 65, 217 (2006).**

75. **M. Tannour-Louet *et al.*, Increased gene copy number of VAMP7 disrupts human male urogenital development through altered estrogen action. *Nature medicine* 20, 715 (Jul, 2014).**

76. **H. O. Goyal *et al.*, Permanent induction of morphological abnormalities in the penis and penile skeletal muscles in adult rats treated neonatally with Diethylstilbestrol or Estradiol Valerate: A dose-response study. *Journal of Andrology* 26, 32 (2005).**

77. H. O. Goyal *et al.*, Estrogen receptor alpha mediates estrogen-inducible abnormalities in the developing penis. *Reproduction* 133, 1057 (May, 2007).
78. E. Willingham *et al.*, Steroid Receptors and Mammalian Penile Development: An Unexpected Role for Progesterone Receptor? *The Journal of Urology* 176, 728 (2006).
79. Y. Satoh *et al.*, Regulation of external genitalia development by concerted actions of FGF ligands and FGF receptors. *Anatomy and embryology* 208, 479 (Sep, 2004).
80. M. L. Gredler, A. W. Seifert, M. J. Cohn, Tissue-specific roles of Fgfr2 in development of the external genitalia. *Development* 142, 2203 (Jun 15, 2015).
81. A. W. Seifert, Z. Zheng, B. K. Ormerod, M. J. Cohn, Sonic hedgehog controls growth of external genitalia by regulating cell cycle kinetics. *Nature communications* 1, 23 (Jun 01, 2010).
82. S. Miyagawa *et al.*, Dosage-dependent hedgehog signals integrated with Wnt/beta-catenin signaling regulate external genitalia formation as an appendicular program. *Development* 136, 3969 (Dec, 2009).
83. Y. Komiya, R. Habas, Wnt signal transduction pathways. *Organogenesis* 4, 68 (2008).
84. B. St-Jacques, M. Hammerschmidt, A. P. McMahon, Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes and Development* 13, 2072 (1999).
85. H. L. Franco *et al.*, Ablation of Indian hedgehog in the murine uterus results in decreased cell cycle progression, aberrant epidermal growth factor signaling, and increased estrogen signaling. *Biol Reprod* 82, 783 (Apr, 2010).
86. A. Eychene, N. Rocques, C. Pouponnot, A new MAFia in cancer. *Nat Rev Cancer* 8, 683 (Sep, 2008).
87. S. Matsushita *et al.*, Androgen Regulates Mafb Expression Through its 3'UTR During Mouse Urethral Masculinization. *Endocrinology* 157, 844 (Feb, 2016).
88. M. SHOZU, K. AKASOFU, TAKENORI HARADA, Y. KUBOTA, A New Cause of Female Pseudohermaphroditism: Placental Aromatase Deficiency. *Journal of Clinical Endocrinology and Metabolism* 72, 560 (1991).
89. K. Yang, Placental 11b-hydroxysteroid dehydrogenase: barrier to maternal glucocorticoids. *Reviews of Reproduction* 197, 129 (1997).
90. W. JP, Estrogen production by fetal rat gonads. *J Steroid Biochem Mol Biol* 44, 459 (1993).
91. B. Vigier *et al.*, Anit-mullerian hormone produces endocrine sex reversal of fetal ovaries. *Proceedings of the National Academy of Sciences of the United States of America* 85, 3684 (1989).
92. D. van der Linde *et al.*, Birth prevalence of congenital heart disease worldwide: a systematic review and meta-analysis. *J Am Coll Cardiol* 58, 2241 (Nov 15, 2011).
93. W. H. Organization, Global registry and database on craniofacial anomalies. *World Health Organization*, Annex 86 (2003).
94. T. Ito *et al.*, Identification of a Primary Target of Thalidomide Teratogenicity. *Science* 327, 1345 (2010).
95. R. L. Brent, Environmental Causes of Human Congenital Malformations: The Pediatrician's Role in Dealing With These Complex Clinical Problems Caused by a Multiplicity of Environmental and Genetic Factors. *Pediatrics* 113, 957 (2004).
96. X. Li *et al.*, Quantification of shape and cell polarity reveals a novel mechanism underlying malformations resulting from related FGF mutations during facial morphogenesis. *Human molecular genetics* 22, 5160 (Dec 20, 2013).
97. J. Jernvall, S. V. E. Kernen, I. Thesleff, Evolutionary modification of development in mammalian teeth: Quantifying gene expression patterns and topography. *PNAS* 97, 14444 (2000).

98. R. M. Green *et al.*, Tfap2a-dependent changes in mouse facial morphology result in clefting that can be ameliorated by a reduction in Fgf8 gene dosage. *Dis Model Mech* 8, 31 (Jan, 2015).
99. M. K. WALKER, R. S. POLLENZ, S. M. SMITH, Expression of the Aryl Hydrocarbon Receptor (AhR) and AhR Nuclear Translocator during Chick Cardiogenesis Is Consistent with 2,3,7,8-Tetrachlorodibenzo-p-dioxin-Induced Heart Defects. *Toxicol and App Pharmac* 143, 407 (1996).
100. M. F. Fernandez *et al.*, Human exposure to endocrine-disrupting chemicals and prenatal risk factors for cryptorchidism and hypospadias: a nested case-control study. *Environmental health perspectives* 115 Suppl 1, 8 (Dec, 2007).
101. R. M. Pratt, L. Dencker, V. M. Diewert, 2,3,7,8-Tetrachlorodi benzo-p-dioxin-Induced Cleft Palate in the Mouse: Evidence for Alterations in Palatal Shelf Fusion. *Teratogenesis, Carcinogenesis, and Mutagenesis* 4, 427 (1984).
102. S. Hernández-Díaz, M. M. Werler, A. M. Walker, A. A. Mitchell, Folic Acid Antagonists during Pregnancy and the Risk of Birth Defects. *N Engl J Med* 343, 1608 (2000).
103. P. D. Winchester, J. Huskins, J. Ying, Agrichemicals in surface water and birth defects in the United States. *Acta Paediatr* 98, 664 (Apr, 2009).
104. J. D. Brender, P. J. Weyer, Agricultural Compounds in Water and Birth Defects. *Curr Environ Health Rep* 3, 144 (Jun, 2016).
105. K. A. Boisen *et al.*, Hypospadias in a cohort of 1072 Danish newborn boys: prevalence and relationship to placental weight, anthropometrical measurements at birth, and reproductive hormone levels at three months of age. *The Journal of clinical endocrinology and metabolism* 90, 4041 (Jul, 2005).
106. N. E. Skakkebaek *et al.*, MALE REPRODUCTIVE DISORDERS AND FERTILITY TRENDS: INFLUENCES OF ENVIRONMENT AND GENETIC SUSCEPTIBILITY. *Physiol Rev* 96, 55 (2016).
107. L. S. Baskin, M. B. Ebbers, Hypospadias: anatomy, etiology, and technique. *Journal of pediatric surgery* 41, 463 (Mar, 2006).
108. A. K. Leung, W. L. Robson, Hypospadias: an update. *Asian J Androl* 9, 16 (Jan, 2007).
109. F. van der Toorn *et al.*, Introducing the HOPE (Hypospadias Objective Penile Evaluation)-score: a validation study of an objective scoring system for evaluating cosmetic appearance in hypospadias patients. *Journal of pediatric urology* 9, 1006 (Dec, 2013).
110. G. H. G. Sinnecker *et al.*, Phenotypic Classification of male pseudohermaphroditism due to steroid 5a-reductase 2 deficiency. *American Journal of Medical Genetics* 63, 223 (1996).
111. N. Kalfa, P. Philibert, L. S. Baskin, C. Sultan, Hypospadias: interactions between environment and genetics. *Mol Cell Endocrinol* 335, 89 (Mar 30, 2011).
112. P. Carbone *et al.*, Cryptorchidism and hypospadias in the Sicilian district of Ragusa and the use of pesticides. *Reprod Toxicol* 22, 8 (Jul, 2006).
113. L. Gaspari *et al.*, Prenatal environmental risk factors for genital malformations in a population of 1442 French male newborns: a nested case-control study. *Human reproduction* 26, 3155 (Nov, 2011).
114. J. Ostby *et al.*, The fungicide procymidone alters sexual differentiation in the male rat by acting as an androgen-receptor antagonist in vivo and in vitro. *Toxicol Ind Health* 15, 80 (1999).
115. A. Bergman, J. J. Heindel, S. Jobling, K. A. Kidd, R. T. Zoeller, State of the Science of Endocrine Disrupting Chemicals- 2012. *WHO*, (2013).
116. Kentaro Suzuki *et al.*, Embryonic development of mouse external genitalia: insights into a unique mode of organogenesis. *Evolution and Development* 4, 133 (2002).
117. P. J. O'Shaughnessy, P. A. Fowler, Endocrinology of the mammalian fetal testis. *Reprod* 141, 37 (Jan, 2011).

118. **R. Murakami, A histological study of the development of the penis of wild-type and androgen-insensitive mice. *J. Anat.* 153, 223 (1986).**
119. **J. Buckley, E. Willingham, K. Agras, L. S. Baskin, Embryonic exposure to the fungicide vinclozolin causes virilization of females and alteration of progesterone receptor expression in vivo: an experimental study in mice. *Environ Health* 5, 4 (Feb 21, 2006).**
120. **J. H. Yang, J. Menshenina, G. R. Cunha, N. Place, L. S. Baskin, Morphology of Mouse External Genitalia: Implications for a Role of Estrogen in Sexual Dimorphism of the Mouse Genital Tubercle. *The Journal of Urology* 184, 1604 (2010).**
121. **C. P. Klingenberg, Evolution and development of shape: integrating quantitative approaches. *Nature reviews. Genetics* 11, 623 (Sep, 2010).**
122. **B. Hallgrimsson *et al.*, Morphometrics, 3D Imaging, and Craniofacial Development. *Curr Top Dev Biol* 115, 561 (2015).**
123. **W. J. Cooper, R. C. Albertson, Quantification and variation in experimental studies of morphogenesis. *Developmental biology* 321, 295 (Sep 15, 2008).**
124. **J. Lleonart, J. Salat, G. J. Torres, Removing allometric effects of body size in morphological analysis. *J Theor Biol* 205, 85 (Jul 7, 2000).**
125. **C. M. Amato, M. Boyd, J. Yang, K. A. McCoy, Organizational effects of the antiandrogen, Vinclozolin, on penis development in the mouse. *Biol Reprod*, (Apr 14, 2018).**
126. **K. Theiler, *The house mouse- Atlas of embryonic development*. (Springer, 1989).**
127. **Miriam Leah Zelditch, D. L. Swiderski, H. D. Sheets, W. L. Fink. (Academic Press, 2004).**
128. **C. Amato, M. Boyd, J. Yang, K. McCoy, Organizational effects of the antiandrogen, Vinclozolin, on penis development in the mouse *Biology of Reproduction*, (2018).**
129. **C. Amato, K. McCoy, A validated protocol to quantify severity of male urogenital feminization using the MOUSE (Mouse objective urethral severity evaluation). *Pediatric research* 80, 880 (2016).**
130. **C. J. Wolf, G. A. LeBlanc, J. S. Ostby, L. E. G. Jr., Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. *Toxicol Sci* 55, 52 (2000).**
131. **L. E. Gray, Jr., J. Ostby, E. Monosson, W. R. Kelce, Environmental antiandrogens: low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. *Toxicol Ind Health* 15, 48 (Jan-Mar, 1999).**
132. **P. M. Foster, M. W. Harris, Changes in androgen-mediated reproductive development in male rat offspring following exposure to a single oral dose of flutamide at different gestational ages. *Toxicol Sci* 85, 1024 (Jun, 2005).**
133. **D. W. Silversides, C. A. Price, G. M. Cooke, Effects of short-term exposure to hydroxyflutamide in utero on the development of the reproductive tract in male mice. *Can. J. Physiol. Pharmacol.* 73, 1582 (1995).**
134. **J. Capdevila, J. C. I. Belmonte, PATTERNING MECHANISMS CONTROLLING VERTEBRATE LIMB DEVELOPMENT. *Annu. Rev. Cell Dev. Biol* 17, 87 (2001).**
135. **C. J. Tabin, Retinoids, Homeoboxes, and Growth Factors: Toward Molecular Models for Limb Development *Cell* 66, 199 (1991).**
136. **A. Zuniga, Next generation limb development and evolution: old questions, new perspectives. *Development* 142, 3810 (Nov 15, 2015).**
137. **D. G. Lupianez *et al.*, Disruptions of topological chromatin domains cause pathogenic rewiring of gene-enhancer interactions. *Cell* 161, 1012 (May 21, 2015).**
138. **L. A. Lettice, P. Devenney, C. De Angelis, R. E. Hill, The Conserved Sonic Hedgehog Limb Enhancer Consists of Discrete Functional Elements that Regulate Precise Spatial Expression. *Cell Rep* 20, 1396 (Aug 8, 2017).**

139. **S. M. Motch Perrine *et al.*, Craniofacial divergence by distinct prenatal growth patterns in Fgfr2 mutant mice. *BMC Dev Biol* 14, 8 (Feb 28, 2014).**
140. **R. Marcucio, B. Hallgrimsson, N. M. Young, Facial Morphogenesis: Physical and Molecular Interactions Between the Brain and the Face. *Curr Top Dev Biol* 115, 299 (2015).**
141. **A. M. Maga, N. Navarro, M. L. Cunningham, T. C. Cox, Quantitative trait loci affecting the 3D skull shape and size in mouse and prioritization of candidate genes in-silico. *Front Physiol* 6, 92 (2015).**
142. **C. Attanasio *et al.*, Fine tuning of craniofacial morphology by distant-acting enhancers. *Science* 342, 1241006 (Oct 25, 2013).**
143. **N. M. Young, H. J. Chong, D. Hu, B. Hallgrimsson, R. S. Marcucio, Quantitative analyses link modulation of sonic hedgehog signaling to continuous variation in facial growth and shape. *Development* 137, 3405 (Oct, 2010).**
144. **J. Cooper, C. Albertson, Quantification and variation in experimental studies of morphogenesis. *Developmental biology* 321, 295 (2008).**
145. **Bergman Å, Heindel JJ, Jobling S, Kidd KA, Zoeller RT, State of the science of endocrine disrupting chemicals - 2012. *W.H.O.*, 63 (2012).**
146. **Kalfa N *et al.*, Mutations of CXorf6 are associated with a range of severities of hypospadias. *European journal of endocrinology / European Federation of Endocrine Societies* 159, 453 (Oct, 2008).**
147. **Manzoni GA, Bracka A, Palminteri E, Marrocco G, Hypospadias surgery: when, what and by whom? *BJU International* 94, 1188 (2004).**
148. **F. van der Toorn *et al.*, Introducing the HOPE (Hypospadias Objective Penile Evaluation)-score: a validation study of an objective scoring system for evaluating cosmetic appearance in hypospadias patients. *J. Pediatr. Urol.* 9, 1006 (Dec, 2013).**
149. **G. R. Cunha, A. Sinclair, G. Risbridger, J. Hutson, L. S. Baskin, Current understanding of hypospadias: relevance of animal models. *Nature reviews. Urology* 12, 271 (May, 2015).**
150. **J. H. Yang, J. Menshenina, G. R. Cunha, N. Place, L. S. Baskin, Morphology of mouse external genitalia: implications for a role of estrogen in sexual dimorphism of the mouse genital tubercle. *J. Urol.* 184, 1604 (Oct, 2010).**
151. **J. Buckley, E. Willingham, K. Agras, L. S. Baskin, Embryonic exposure to the fungicide vinclozolin causes virilization of females and alteration of progesterone receptor expression in vivo: an experimental study in mice. *Environ. Health.* 5, 4 (2006).**
152. **C. V. Rider, J. Furr, V. S. Wilson, L. E. Gray, Jr., A mixture of seven antiandrogens induces reproductive malformations in rats. *Int. J. Androl.* 31, 249 (Apr, 2008).**
153. **C. M. Rocheleau, P. A. Romitti, L. K. Dennis, Pesticides and hypospadias: a meta-analysis. *J. Pediatr. Urol.* 5, 17 (Feb, 2009).**
154. **K. Suk Kim *et al.*, Induction of hypospadias in a murine model by maternal exposure to synthetic estrogens. *Environ. Res.* 94, 267 (2004).**
155. **D. W. Silversides;, C. A. Price;, G. M. Cooke, Effects of Short-Term Exposure to Hydroxyflutamide in utero on the development of the Reproductive Tract in Male Mice *Canadian J. of Physio. and Pharmacol.* 73, 1582 (1995).**
156. **L. F. van der Zanden *et al.*, Aetiology of hypospadias: a systematic review of genes and environment. *Human reproduction update* 18, 260 (May-Jun, 2012).**
157. **S. L. Carmichael *et al.*, Diacylglycerol kinase K variants impact hypospadias in a California study population. *J. Urol.* 189, 305 (Jan, 2013).**
158. **T. Ogata, J. Laporte, M. Fukami, MAMLD1 (CXorf6): a new gene involved in hypospadias. *Hormone research* 71, 245 (2009).**

159. A. E. Czeizel, The primary prevention of birth defects: Multivitamins or folic acid? *Int. J. Med. Sci.* 1, 50 (2004).
160. S. Mavrogenis, R. Urban, A. E. Czeizel, N. Acs, Possible preventive effect of high doses of folic acid for isolated hypospadias: a national population-based case-control study. *American journal of medical genetics. Part A* 164A, 3108 (Dec, 2014).
161. G. Ormond *et al.*, Endocrine disruptors in the workplace, hair spray, folate supplementation, and risk of hypospadias: case-control study. *Environ. Health Perspect.* 117, 303 (Feb, 2009).
162. Kelce WR, Monosson E, Gamcsik MP, Laws SC, Gray LE, Environmental hormone disruptors evidence that vinclozolin developmental toxicity is mediated by metabolites. *Toxicol. and App. Pharmacol.* 126, 276 (1994).
163. Bates D, Machler M, Bolker BMM, and Walker SC, Fitting linear mixed-effects models using lme4. *R News*, 27 (2005).
164. J. Fox, Effect displays in R for generalised linear models. *Journal of Statistical Software* 8, 1 (2003).
165. A. C. Gore *et al.*, EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. *Endocr Rev* 36, E1 (Dec, 2015).
166. L. Lund *et al.*, Prevalence of hypospadias in Danish boys: a longitudinal study, 1977-2005. *European urology* 55, 1022 (May, 2009).
167. N. Nassar, C. Bower, A. Barker, Increasing prevalence of hypospadias in Western Australia, 1980-2000. *Archives of disease in childhood* 92, 580 (Jul, 2007).
168. D. J. P. Barker, The developmental origins of adult disease. *Eur J Epidemiol* 18, 733 (2003, 2003).
169. P. Pihlajamaa, B. Sahu, O. A. Janne, Determinants of Receptor- and Tissue-Specific Actions in Androgen Signaling. *Endocr Rev* 36, 357 (Aug, 2015).
170. M. M. McCarthy, Estradiol and the developing brain. *Physiol Rev* 88, 91 (Jan, 2008).
171. M. D. McAbee, L. L. DonCarlos, Estrogen, But Not Androgens, Regulates Androgen Receptor Messenger Ribonucleic Acid Expression in the Developing Male Rat Forebrain. *Endocrinol* 140, 3674 (1999).
172. J. M. Green *et al.*, Early life exposure to ethinylestradiol enhances subsequent responses to environmental estrogens measured in a novel transgenic zebrafish. *Sci Rep* 8, 2699 (Feb 9, 2018).
173. K. Bay, C. Asklund, N. E. Skakkebaek, A. M. Andersson, Testicular dysgenesis syndrome: possible role of endocrine disrupters. *Best Pract Res Clin Endocrinol Metab* 20, 77 (2006).
174. N. E. Skakkebaek, E. Rajpert-De Meyts, K. M. Main, Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Human reproduction* 16, 972 (2001).
175. L. Baskin *et al.*, Urethral seam formation and hypospadias. *Cell Tissue Res* 305, 379 (2001).
176. J. M. Molina-Molina *et al.*, Steroid receptor profiling of vinclozolin and its primary metabolites. *Toxicology and applied pharmacology* 216, 44 (Oct 1, 2006).
177. P. Murugesan, T. Muthusamy, K. Balasubramanian, J. Arunakaran, Effects of vitamins C and E on steroidogenic enzymes mRNA expression in polychlorinated biphenyl (Aroclor 1254) exposed adult rat Leydig cells. *Toxicology* 232, 170 (Apr 11, 2007).
178. J. Ostby, E. Monosson, W. R. Kelce, L. E. Gray, Environmental antiandrogens: low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. *Toxicol. and Indust. Health* 15, 48 (1999).
179. G. L. Humason, *Animal Tissue Techniques*. (Johns Hopkins University Press, Baltimore, Maryland, ed. 5th, 1997).
180. . (R Foundation for Statistical Computing, Vienna, Austria, 2016).

181. B. M. Bolker *et al.*, Generalized linear mixed models: a practical guide for ecology and evolution. *Trends in ecology & evolution* 24, 127 (Mar, 2009).
182. S.-F. Lu, S. E. Mckenna, A. Cologer-Clifford, E. A. Nau, N. G. Simom, Androgen receptor in mouse brain: Sex Differences and similarities in autoregulation. *Endocrinol* 139, 1594 (1998).
183. F. M. Bentvelsen; *et al.*, Developmental pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat. *Mol Cell Endocrinol*, 245 (1995).
184. J. J. Winston, R. E. Meyer, M. E. Emch, Geographic Analysis of Individual and Environmental Risk Factors for Hypospadias Births. *Birth Defects Res* Part A, 887 (2014).
185. C. Lane *et al.*, A population-based study of prevalence trends and geospatial analysis of hypospadias and cryptorchidism compared with non-endocrine mediated congenital anomalies. *Journal of pediatric urology* 13, 284 e1 (Jun, 2017).
186. M. Manikkam, R. Tracey, C. Guerrero-Bosagna, M. K. Skinner, Pesticide and insect repellent mixture (permethrin and DEET) induces epigenetic transgenerational inheritance of disease and sperm epimutations. *Reprod Toxicol* 34, 708 (Dec, 2012).
187. H. Levine *et al.*, Temporal trends in sperm count: a systematic review and meta-regression analysis. *Human reproduction update*, 1 (2017).
188. M. S. Bloom *et al.*, Associations between urinary phthalate concentrations and semen quality parameters in a general population. *Human reproduction* 30, 2645 (Nov, 2015).
189. Y. H. Chiu *et al.*, Intake of Fruits and Vegetables with Low-to-Moderate Pesticide Residues Is Positively Associated with Semen-Quality Parameters among Young Healthy Men. *The Journal of nutrition* 146, 1084 (May, 2016).
190. L. J. Paulozzi, J. D. Erickson, R. J. Jackson, Hypospadias Trends in Two US Surveillance Systems. *Pediatr* 100, 831 (1997).
191. CDC, NBDPN, Major birth Defects from Population-based birth defects surveillance programs in the United States, 2009-2013. *Birth Defects Research (Part A)* 106, S1 (2016).
192. P. D. Mouriquand *et al.*, Surgery in disorders of sex development (DSD) with a gender issue: If (why), when, and how? *Journal of pediatric urology* 12, 139 (Jun, 2016).
193. A. Bracka, Sexuality after hypospadias repair. *BJU International* 83, 29 (1999).
194. P. J. Lioy, Assessing total human exposure to contaminents. *Environ Sci Technol Lett* 24, 938 (1990).
195. G. Ormond *et al.*, Endocrine disruptors in the workplace, hair spray, folate supplementation, and risk of hypospadias: case-control study. *Environ Health Perspect* 117, 303 (Feb, 2009).
196. F. P. Perera, W. Jedrychowski, V. Rauh, R. M. Whyatt, Molecular epidemological research on the effects of environmental pollutants on the fetus. *Environmental health perspectives* 107, 451 (1999).
197. A. Lucas, M. S. Fewtrell, T. J. Cole, Fetal origins of adult disease—the hypothesis revisited. *BMJ* 319, 245 (1999).
198. J. S. Xing, Z. M. Bai, Is testicular dysgenesis syndrome a genetic, endocrine, or environmental disease, or an unexplained reproductive disorder? *Life Sci* 194, 120 (Feb 1, 2018).
199. M. A. Hanson, P. D. Gluckman, Developmental Origins of Health and Disease: New Insights. *Basic and Clinical Pharmacology and Toxicology* 102, 90 (2007).
200. J. C. DeWitt, H. B. Patisaul, Endocrine disruptors and the developing immune system. *Current Opinion in Toxicology* 10, 31 (2018).
201. N. A. Philbrook, L. M. Winn, Sub-chronic sulforaphane exposure in CD-1 pregnant mice enhances maternal NADPH quinone oxidoreductase 1 (NQO1) activity and mRNA expression of NQO1, glutathione S-transferase, and glutamate-cysteine ligase: potential implications for fetal protection against toxicant exposure. *Reprod Toxicol* 43, 30 (Jan, 2014).

202. **S. Jahan, A. Zahra, U. Irum, N. Iftikhar, H. Ullah, Protective effects of different antioxidants against cadmium induced oxidative damage in rat testis and prostate tissues. *Systems biology in reproductive medicine* 60, 199 (Aug, 2014).**

203. **C. A. Houghton, R. G. Fassett, J. S. Coombes, Sulforaphane and Other Nutrigenomic Nrf2 Activators: Can the Clinician's Expectation Be Matched by the Reality? *Oxid Med Cell Longev* 2016, 7857186 (2016).**

204. **K. Itoh *et al.*, Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes and Development* 13, 76 (1998).**

205. **R. K. Thimmulappa *et al.*, Identification of Nrf2-regulated Genes Induced by the Chemopreventive Agent**
Sulforaphane by Oligonucleotide Microarray. *Cancer Research* 62, 5196 (2002).

206. **Z. Qin *et al.*, Protective effects of sulforaphane on di-n-butyl phthalate induced testicular oxidative stress injury in male mice offsprings via activating Nrf2/ARE pathway. *Oncotarget* 8, 82956 (2017).**

207. **N. Juge, R. F. Mithen, M. Traka, Molecular basis for chemoprevention by sulforaphane: a comprehensive review. *Cell Mol Life Sci* 64, 1105 (May, 2007).**

208. **N. B. D. P. Network, Major birth Defects from Population-based birth defects surveillance programs in the**
United States, 2009-2013. *Birth Defects Research (Part A)* 106, S1 (2016).

209. **G. Barbagli, M. De Angelis, E. Palminteri, M. Lazzeri, Failed hypospadias repair presenting in adults. *European urology* 49, 887 (May, 2006).**

210. **J. Adams, A. Bracka, Reconstructive surgery for hypospadias: A systematic review of long-term patient satisfaction with cosmetic outcomes. *Indian J Urol.* 32, 93 (2016).**

211. **P. J. Hynes, J. P. Fraher, The development of the male genitourinary system: III. The formation of the spongiose and glandar urethra. *Br J Plast Surg* 57, 203 (Apr, 2004).**

212. **E. L. Abel *et al.*, Sulforaphane induces phase II detoxication enzymes in mouse skin and prevents mutagenesis induced by a mustard gas analog. *Toxicology and applied pharmacology* 266, 439 (Feb 1, 2013).**

213. **X. Shi, B. Zhou, The role of Nrf2 and MAPK pathways in PFOS-induced oxidative stress in zebrafish embryos. *Toxicol Sci* 115, 391 (Jun, 2010).**

214. **A. Enomoto *et al.*, High Sensitivity of Nrf2 Knockout Mice to Acetaminophen Hepatotoxicity Associated with Decreased Expression of ARE- Regulated Drug Metabolizing Enzymes and Antioxidant Genes. *Toxicol Sci* 59, 169 (2001).**

215. **G. P. Sykiotis, D. Bohmann, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. *Dev Cell* 14, 76 (Jan, 2008).**

216. **J. H. An, T. K. Blackwell, SKN-1 links C. elegans mesendodermal specification to a conserved oxidative stress response. *Genes Dev* 17, 1882 (Aug 1, 2003).**

217. **T. Rangasamy *et al.*, Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. *J Exp Med* 202, 47 (Jul 4, 2005).**

218. **T. O. Khor *et al.*, Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-induced colitis. *Cancer Res* 66, 11580 (Dec 15, 2006).**

219. **T. Iizuka *et al.*, Nrf2-deficient mice are highly susceptible to cigarette smoke-induced emphysema. *Genes Cells* 10, 1113 (Dec, 2005).**

220. **B. Yu *et al.*, Genetic variation in the Nrf2 promoter associates with defective spermatogenesis in humans. *J Mol Med (Berl)* 90, 1333 (Nov, 2012).**

221. **B. Yu *et al.*, Cigarette smoking is associated with human semen quality in synergy with functional NRF2 polymorphisms. *Biol Reprod* 89, 5 (Jul, 2013).**

222. **J. M. Marzec *et al.*, Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury. *FASEB* 21, 2237 (2007).**
223. **A. T *et al.*, Nrf2 gene promoter polymorphism is associated with ulcerative colitis in a Japanese population. *Hepato-gastroenterology* 55, 394 (2008).**
224. **C.-C. Hong *et al.*, Genetic variability in iron-related oxidative stress pathways (Nrf2, NQ01, NOS3, and HO-1), iron intake, and risk of postmenopausal breast cancer. *Cancer Epidemiol Biomarkers Prev* 16, (2007).**
225. **Y. Shinkai, D. Sumi, I. Fukami, T. Ishii, Y. Kumagai, Sulforaphane, an activator of Nrf2, suppresses cellular accumulation of arsenic and its cytotoxicity in primary mouse hepatocytes. *FEBS letters* 580, 1771 (Mar 20, 2006).**
226. **X. Chen, J. Liu, S. Y. Chen, Sulforaphane protects against ethanol-induced oxidative stress and apoptosis in neural crest cells by the induction of Nrf2-mediated antioxidant response. *British journal of pharmacology* 169, 437 (May, 2013).**
227. **K. Itoh *et al.*, An Nrf2/Small Maf heterodimer mediates the induction of Phase II detoxifying enzyme genes through antioxidant response elements. *Biochemical and Biophysical Research Communications* 236, 313 (1997).**
228. **A. Sierra-Santoyo, G. Castaneda-Hernandez, R. A. Harrison, H. A. Barton, M. F. Hughes, Pharmacokinetics and dosimetry of the antiandrogen vinclozolin after oral administration in the rat. *Toxicological sciences : an official journal of the Society of Toxicology* 106, 55 (Nov, 2008).**
229. **F. G. de Oca, L. Lopez-Gonzalez Mde, D. C. Escobar-Wilches, R. Chavira-Ramirez, A. Sierra-Santoyo, Vinclozolin modulates hepatic cytochrome P450 isoforms during pregnancy. *Reproductive toxicology* 53, 119 (Jun, 2015).**
230. **Kelce WR, Monosson E, Gamcsik MP, Laws SC, Gray LE, Environmental hormone disruptors evidence that vinclozolin developmental toxicity is mediated by metabolites. *Toxicology and applied pharmacology* 126, 276 (1994).**
231. **V. Yoxall *et al.*, Modulation of hepatic cytochromes P450 and phase II enzymes by dietary doses of sulforaphane in rats: Implications for its chemopreventive activity. *International journal of cancer* 117, 356 (Nov 10, 2005).**
232. **R. E. CHAPIN *et al.*, Are Mouse Strains Differentially Susceptible to the Reproductive Toxicity of Ethylene Glycol Monomethyl Ether? A Study of Three Strains. *Fundamental and Applied Toxicology* 21, 8 (1993).**
233. **Q. Zhang, J. Pi, C. G. Woods, M. E. Andersen, Phase I to II cross-induction of xenobiotic metabolizing enzymes: a feedforward control mechanism for potential hormetic responses. *Toxicol Appl Pharmacol* 237, 345 (Jun 15, 2009).**
234. **N. Hanlon *et al.*, Absolute bioavailability and dose-dependent pharmacokinetic behaviour of dietary doses of the chemopreventive isothiocyanate sulforaphane in rat. *Br J Nutr* 99, 559 (Mar, 2008).**
235. **N. Kalfa *et al.*, Is Hypospadias Associated with Prenatal Exposure to Endocrine Disruptors? A French Collaborative Controlled Study of a Cohort of 300 Consecutive Children Without Genetic Defect. *European urology* 68, 1023 (Dec, 2015).**
236. **A. L. Brantsaeter *et al.*, Organic Food Consumption during Pregnancy and Hypospadias and Cryptorchidism at Birth: The Norwegian Mother and Child Cohort Study (MoBa). *Environmental health perspectives* 124, 357 (Mar, 2016).**
237. **A. Sierra-Santoyo, G. Castaneda-Hernandez, R. A. Harrison, H. A. Barton, M. F. Hughes, Pharmacokinetics and dosimetry of the antiandrogen vinclozolin after oral administration in the rat. *Toxicol Sci* 106, 55 (Nov, 2008).**

238. C. Amato, K. McCoy, The morphogenic, hormonal, and gene expression changes controlling external genitalia development. *In-Review*, (2018).
239. C. Wong, W. Kelce, M. Sar, E. Wilson, Androgen receptor antagonist versus angaonist activities of the fungicide vinclozolin relative to hydroxyflutamide. *The Journal of biological chemistry* 270, 19998 (1995).
240. M. Stewart, A. Pask, In utero exposure to both high and low dose diethylstilbestrol disrupts mouse genital tubercle development. *Biology of Reproduction*, (2018).
241. C. Mamoulakis *et al.*, Seasonal trends in the prevalence of hypospadias: Aetiological implications. *Experimental and therapeutic medicine* 13, 2960 (Jun, 2017).
242. M. Asakage *et al.*, Sulforaphane induces inhibition of human umbilical vein endothelial cells proliferation by apoptosis. *Angiogenesis* 9, 83 (2006).
243. S. Radice, L. Marabini, M. Gervasoni, M. Ferraris, E. Chiesara, Adaptation to oxidative stress: effects of vinclozolin and iprodione on the HepG2 cell line. *Toxicology* 129, 183 (1998).
244. R. Gupta, G. ES, D. BK, T. AS, A. J, Vitamin C and Vitamin E protect the rat testes from cadmium-induced reactive oxygen species *Mol. Cells* 17, 132 (2004).

Appendix: Animal Use Protocol

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

October 20, 2016

Krista McCoy, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. McCoy:

Your Animal Use Protocol entitled, "Developing a Nutritive Therapy for Reducing the Severity and Incidence of Hypospadias" (AUP #D297a) was reviewed by this institution's Animal Care and Use Committee on October 20, 2016. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION NOVEMBER, 2013

Project Title:

Developing a nutritive therapy for reducing the severity and incidence of hypospadias.

	Principal Investigator	Secondary Contact
Name	Krista A. McCoy	Ciro Amato
Dept.	Biology	Biology
Office Ph #	252-737-2730	Click here to enter text.
Cell Ph #	571-315-2884	239-825-7829
Pager #	Click here to enter text.	Click here to enter text.
Home Ph #	262-565-5780	Click here to enter text.
Email	mccoyk@ecu.edu	Amatoc13@students.ecu.edu

For IACUC Use Only

AUP # D297a			
New/Renewal Renewal 10/20/16			
Full Review/Date	DR/Date		
Approval Date 10/20/16			
Study Type reproductive - chemicals			
Pain/Distress Category D			
Surgery	Survival	Multiple	
Prolonged Restraint			
Food/Fluid Regulation			
Other			
Hazard Approval/Dates	Rad	IBC	EHS
OHP Enrollment			Vinclozolin,
Mandatory Training			sulforaphane,
Amendments Approved			corn oil, Resveratol,
			D-carnosine

- single housing
- Howell 5112A & Ragsdale Annex