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Developmental Exposure to Xenoestrogens: Effects on the Mouse Mammary Gland Development and Response to Estrogen

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DEVELOPMENTAL EXPOSURE TO XENOESTROGENS: EFFECTS ON THE MOUSE MAMMARY GLAND DEVELOPMENT AND RESPONSE TO ESTROGEN

A Thesis Presented

by

SRIDURGADEVI KOLLA

Submitted to the Graduate School of the
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DEDICATION

To all the CD-1 mice.

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ABSTRACT

DEVELOPMENTAL EXPOSURE TO XENOESTROGENS: EFFECTS ON THE MOUSE MAMMARY GLAND DEVELOPMENT AND RESPONSE TO ESTROGEN

MAY 2018

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Humans experience ubiquitous exposures to estrogenic environmental chemicals from food, personal care products, and other industrial and consumer goods. Bisphenol A (BPA), a well-studied xenoestrogen, is known to alter development of estrogen-sensitive organs including the brain, reproductive tract, and mammary gland. Bisphenol S (BPS), which has a similar chemical structure to BPA, is also used in many consumer products, but its effects on estrogen-sensitive organs in mammals has not been thoroughly examined. In our study, pregnant CD-1 mice were orally exposed to BPS or ethinyl estradiol (EE2, a positive control for estrogenicity) from gestational day 9 through postnatal day (PND) 2, the period when many estrogen-sensitive organs are developing. After weaning, the offspring were administered either oil (vehicle) or an estrogen challenge (1 μg EE2/kg/day) for ten days starting at PND21 (prior to puberty), PND80 (early adulthood), or PND260 (later adulthood). Timing of puberty was evaluated in females by noting the date on which vaginal opening occurred. After the 10 day estrogen challenge, we evaluated the response of endocrine sensitive organs through measurements of organ weight, tissue morphology, and gene expression in both males and females. We observed dose- and sex-specific effects

of BPS and EE2 treatment, as well as alterations in the responses of males and females to the estrogen challenge. This study sheds light on the effects of low dose xenoestrogen exposures on estrogen-sensitive organs including the reproductive tract and mammary gland. Furthermore, it improves our understanding of the influence of environmental chemicals on secular trends of earlier age of puberty in girls reported over the past few decades.

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BACKGROUND

Introduction

Endocrine Disrupting Chemicals

In the mid-1900s there was a significant rise in the production of man-made chemicals. Industrialization of synthetic chemical manufacturing and their use in consumer products was popular and common. The use of chemicals in everyday products brought ease and comfort to a growing and fast paced society. The manufacturers of these chemicals did not foresee their latent harmful effects on wildlife and humans.

The publication of Rachel Carson's *Silent Spring* in 1962, where she discussed the adverse effects of pesticide DDT and other widely used chemicals, sparked the environmental movement and a sense of awareness of the threat that these chemicals could pose to the world [1]. Although work by environmental health scientists and ecologists built on the work of Carson and others, it wasn't until 1991 that the focus on the effects of these chemicals on the endocrine system gained widespread attention [2]. At that time, a group of scientists from interdisciplinary fields congregated at the Wingspread Conference Center to discuss the effects of hormonally active environmental chemicals on humans, laboratory animals, and wildlife. At the Wingspread Conference, these scientists came to the consensus that these chemicals were capable of inducing harmful effects on the endocrine system. It was at this conference, organized by Theo Colborn, where the term 'endocrine disruptor' was coined. This was the start of the field of endocrine disruption.

The Endocrine Society defines Endocrine Disrupting Chemicals (EDCs) as "exogenous chemical(s), or mixtures of chemicals, that interfere with any aspect of hormone action" [3]. Many are man-made and used in pesticides, personal care products, food and food packaging, and industrial solvents. In 2013, the UNEP/WHO State of the Science document on EDCs estimated that there about 800 chemicals suspected of interfering with the endocrine system [4, 5].

Public health scientists have noted a significant rise in non-communicable endocrine related diseases in the past few decades [5, 6]. This rapid increase cannot be due to altered genetics alone; rather, it is likely that environmental factors are associated with many of these diseases. Over an individual's lifespan, the body goes through many developmental and physiological changes that are dependent on hormones [7]. Because EDCs can mimic, block, or otherwise interfere with hormone signaling, exposures to these compounds, particularly during vulnerable periods of development, can interfere with major physiological processes that can be detrimental to the health of the individual and future generations. In addition to the importance of timing, both duration and dose of EDC exposure can result in varying adverse effects. There are many well-studied chemicals that are known EDCs, including DDT, PAHs, PCBs, BPA, and phthalates. Studies have shown that EDCs affect male and female reproduction and neurodevelopment, and can induce cancer, obesity, and other non-communicable diseases and disorders [6].

It has become widely acknowledged that EDCs are capable of disrupting the development and health of human and wildlife populations [4, 6]. Yet, many factors can interfere with the evaluation of chemicals for regulatory purposes. Current approaches to hazard identification and risk assessment of environmental contaminants are based on

traditional regulatory toxicity testing, using standardized testing guidelines, which are outdated and do not properly evaluate endocrine disease vulnerability [7, 8]. Hazard and risk assessments also rely on historical assumptions related to linear dose-responses, the selection of doses for testing, and 'gold-standard' endpoints. To address these gaps, regulatory agencies will likely need to embrace a more holistic, evidence-based, and biologically relevant approach when evaluating the safety of synthetic chemicals.

Diethylstilbestrol (DES): the first synthetic endocrine disruptor

In 1938, Charles Dodds and colleagues synthesized diethylstilbestrol (DES), the first orally active synthetic estrogen [9]. It quickly became popular for use as a pharmaceutical and was prescribed for several purposes including as an oral contraceptive, to treat hot flashes in menopausal women, and to block lactation in postpartum mothers [10]. From 1940 to 1970, DES was prescribed to approximately 2 million women under the mistaken notion that it would prevent miscarriages and would promote the growth of bigger and healthier babies [11]; there was no evidence available that increased estrogen treatment would be beneficial to maintain pregnancies or that it would improve the health and development of the fetus.

Remarkably, the DES daughters (e.g., females exposed in the womb) were born without any obvious signs of harm or disease, allowing exposures to continue for several decades [11]. Yet, once these DES-daughters reached puberty, they were more likely to develop a rare cancer, clear cell adenocarcinoma of the vagina [12, 13]. Furthermore, longitudinal studies of DES daughters that evaluated these women in adulthood found that they were at increased risk to develop preeclampsia and infertility, and were more

likely to reach menopause at an earlier age [14, 15]. Furthermore, 90-95% of exposed daughters were found to have reproductive tract malformations, reproductive dysfunction, and poor pregnancy outcomes including ectopic pregnancies and premature births [16]. Finally, because breast cancer has a latency of several decades, studies conducted when DES daughters reached 40+ years of age determined that these women have an increased risk of developing breast cancer [13, 17].

Additional studies have revealed that DES negatively impacted the women exposed during pregnancy (e.g., DES mothers) with increased risk of breast cancer [18]; the relative risk for DES mothers was lower than the relative risk in DES daughters, consistent with an increased vulnerability in females during embryonic and fetal development. Compelling studies have also revealed effects of DES on future generations including DES-granddaughters, exposed as germ cells in their gestating mothers [19, 20].

The story of DES provides insight into the potential (and often still undiscovered) adverse effects of anthropogenic estrogens in our environment today — many of which lack proper testing and evaluation by regulatory agencies. DES also illustrated that the effects of developmental estrogen exposures can be latent, and only visibly manifest when additional hormone exposures (e.g., the increased estrogens associated with puberty) occur [21].

BPA, a well-studied estrogenic EDC

Bisphenol A (BPA) is an estrogenic EDC (Figure 1.1) that has received wide attention from scientists in numerous disciplines as well as physicians, health advocates, and the public [22]. BPA is used in many everyday consumer products including food and

beverage containers, plastics, thermal paper, toys, medical equipment, and others [23]. In 2008, 2.7 billion kg of BPA was produced worldwide for manufacturing goods and most of it is released into the environment either indirectly or in consumer products. Its widespread use has become a major public health concern for both the environment and human health. BPA is detected in human urine, serum, saliva, maternal and fetal plasma, amniotic fluid, and placental tissue [24].

A large number of human epidemiological studies have examined associations between BPA exposures and human health conditions [25]. Many studies have shown that BPA exposures are associated with health problems including altered reproductive performance, obesity, diabetes, hyperactivity, impaired cognitive abilities, and much more. These studies have been challenged because of limitations in their study designs and exposure assessments; BPA is rapidly metabolized, and it is difficult to draw conclusions about typical daily exposures from a single spot urine sample. Although many of these studies have had cross-sectional designs, making it difficult to establish causal relationships between BPA exposures and disease, a small number of prospective studies support the conclusion that BPA can alter endocrine function and contribute to dysfunction in exposed humans.

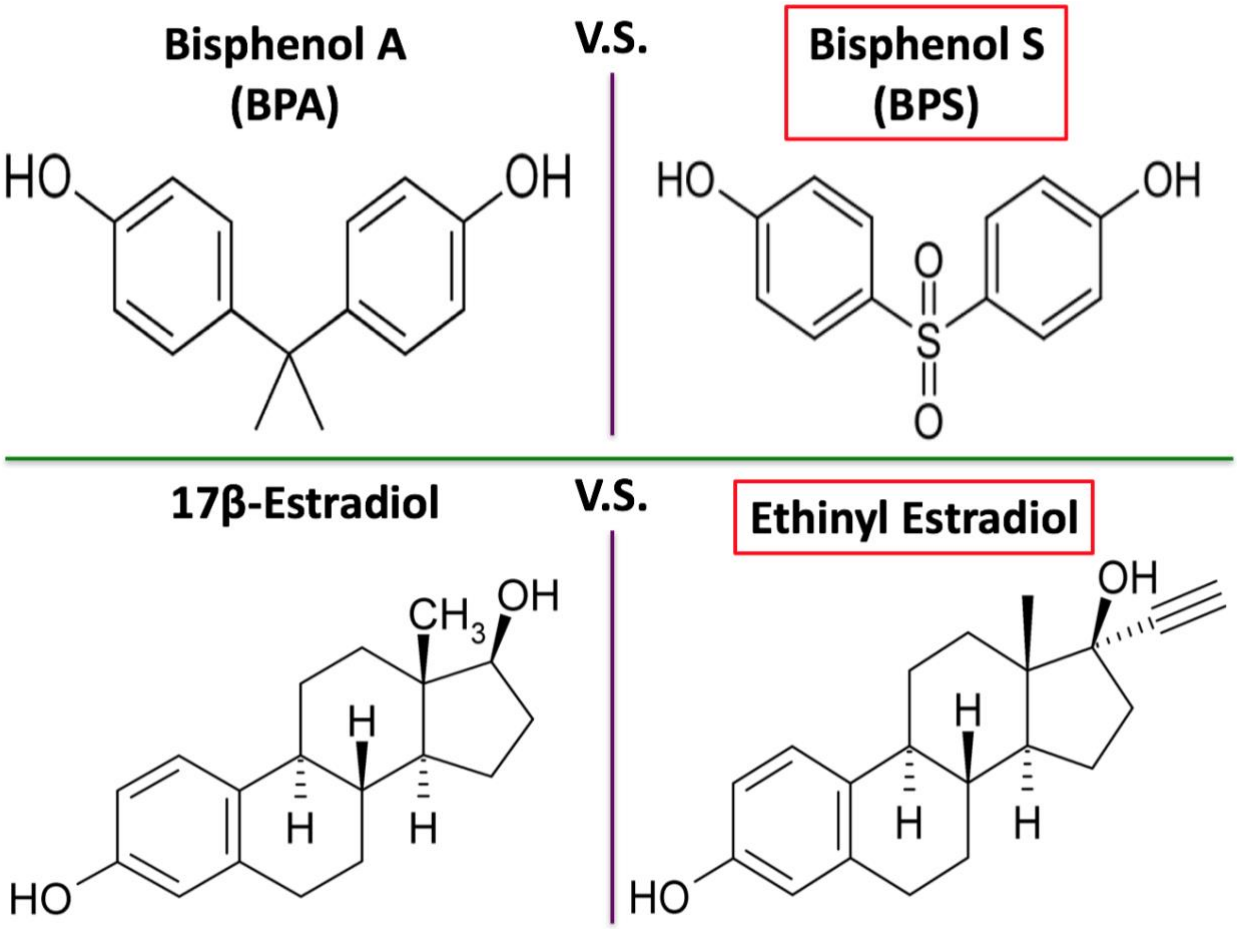

Figure 1.1. Chemical structures of BPA, BPS, 17β-estradiol and 17α-ethinyl estradiol.

Animal studies using controlled experimental protocols have evaluated many of these same outcomes as well as others [26]. Hundreds of studies, many examining low doses of BPA, show that BPA can alter development of organs including the brain, reproductive tract, liver and kidneys; induce cancers of the prostate and mammary gland; alter the animal's responsiveness to hormones, allergens and carcinogens; alter neurobehaviors; and induce multiple aspects of metabolic syndrome. Most animal studies have focused on rodents exposed during critical periods of development including gestation and perinatal life, but a small number of studies have also revealed effects on the F2

generation (exposed as germ cells) and beyond, suggesting that this compound may induce transgenerational effects [26].

BPA mimics estrogen in both cultured cells and live animals [27]. To measure estrogenic activity, an *in vitro* assay called the E-SCREEN, which is highly sensitive to estrogen, was used to measure cell proliferation in the MCF7 breast epithelial cell line following exposure to a wide range of concentrations of BPA [28]. This assay can differentiate between partial and full estrogen receptor (ER) agonists (which partially or fully mimic the actions of estrogens), but it can also be used to identify ER antagonists (which block the actions of endogenous estrogens) [29]. The E-SCREEN has been used to verify the estrogenic properties of BPA. *In vivo* assays of estrogenicity such as the uterotrophic assay have also confirmed BPA's ER agonist properties [30]. In the ovariectomized rat model, BPA can induce responses in a range of estrogen-sensitive endpoints including uterine weight, height of the uterine epithelial cells, uterine expression of the estrogen-inducible protein lactoferrin, and proliferation of uterine epithelial cells [31].

One remarkable feature of BPA is its ability to act at low doses. In rodents, exposures as low as 25 ng/kg/day have been shown to disrupt development of estrogen-sensitive organs including the brain and mammary gland [32]. Several studies have investigated the mechanisms by which BPA can induce responses at low concentrations; these studies, revealing effects at low doses, often in the part-per-billion concentration range, show that BPA can have equal potency as estradiol for some endpoints [33]. This means that the former characterization of BPA as a "weak" estrogen is not accurate, at least

for some endpoints. It has, in fact, become well understood in the endocrinology community that potency is specific to the endpoint examined [8].

Although most concern surrounding BPA is based on its known ability to mimic natural estrogens, it has other endocrine disrupting properties [22]. BPA not only binds to the nuclear ERs, but also membrane ER and estrogen related receptor γ. It also disrupts signaling through androgen receptor and thyroid hormone receptor.

BPS, an Alternative to BPA

After numerous studies showed negative adverse effects from BPA, the public began to raise concern about the inclusion of an estrogenic compound in consumer goods. In particular, consumer advocacy groups pushed back against the inclusion of BPA in products designed for babies and children including baby bottles and sippy cups. In response to these consumer demands, product manufacturers began removing BPA from products, labeling them "BPA-free" [34]. What was not anticipated was the substitution of BPA with other chemicals, like BPS, which has a similar chemical structure, and likely can induce similar biological effects. BPS is now used in baby bottles, thermal receipt paper and other paper products; it has also been detected in canned foods [35-38]. Biomonitoring studies from the US and Asian countries indicate that human exposures to BPS are low but widespread, vary by location [39] and have increased over the last decade [40].

In the past few years, studies have revealed that popular BPA replacements, including BPS, have estrogenic activities both *in vitro* and *in vivo* [41]. It is also possible

that BPS has various other concerning properties that are not induced by BPA, including alterations in progesterone signaling [42].

Compared to the large number of published studies examining the effects of BPA, relatively few have characterized the effects of BPA-replacement chemicals on laboratory animals. In one study, a low dose of BPS was sufficient to induce the birth of new neurons (via the process known as neurogenesis) in the hypothalamus of exposed zebrafish. The effects of BPS were similar to those observed after exposure to BPA [43, 44]. Boucher and colleagues have also shown that BPS increases the rate of adipocyte differentiation from human preadipocytes, provokes lipid production in these cells, and increases the expression of several adipogenic markers [45, 46]. Finally, a study performed in female rats revealed that low doses of BPS can alter function of the heart at the whole organ, cellular, and protein levels of biological organization [47].

Ongoing work in the Vandenberg lab has evaluated the effects of low dose BPS exposure on CD-1 mice exposed either during pregnancy/lactation [48]. Exposure to BPS induced alterations in pup retrieval and time the mothers spent on the nest. Brain regions important for the onset of maternal behavior were also affected following BPS treatment. Additional studies have shown that exposures to BPS during pregnancy and lactation alter the dam's mammary gland, inducing early involution, with effects on growth and development of offspring [49].

Maternal behaviors were also affected in females exposed to BPS during perinatal development [48]. Some of these abnormal behaviors were severe including increased rates of infanticide and neglect. Mice exposed to low doses of BPS during early development also displayed abnormalities in uterine weight, body weight, and social behaviors [50-52].

Other effects of BPS, including on the mammary gland, are described in greater detail in Chapter 2. Collectively, these results reveal that environmental exposures to BPS during vulnerable stages of life can influence estrogen-sensitive organs and endpoints, with some effects that are not observed until long after exposures cease.

BPS is widely considered a "Regrettable Replacement". Regrettable substitutions occur when harmful chemicals are replaced with other potentially harmful chemicals, often with fewer hazard evaluations available [34]. When this occurs, it calls into question the strength and quality of chemical regulations worldwide. The public is often left to wonder why there is minimal testing on chemicals that are used in many different kinds of consumer products, allowing for widespread exposures of the population including vulnerable individuals. Studying the hazards associated with the replacements will hopefully contribute to appropriate changes to the chemical regulation processes.

EE_2, an Oral Contraceptive and positive control for estrogenicity

Ethinyl Estradiol (EE2) is a pharmaceutical estrogen that is widely used in oral contraceptives by more than 100 million women worldwide [53, 54]. The Centers for Disease and Control (CDC) reported in 2012 that approximately 10.2 million women were using the pill as a form of contraceptive in the US. Approximately 9% of women will conceive within the first year of using oral contraceptives, mostly due to missed doses. In the US, ~2 million women become pregnant each year while using oral contraceptives [55], with approximately 3% of pregnant women reporting that they took estrogenic pharmaceuticals during their first trimester [56]. These results suggest that a significant proportion of live births involve offspring that were exposed to this synthetic estrogen for

some period of gestation. A recent study capitalizing on the availability of extensive birth registries in Denmark questioned whether oral contraceptive use before or during pregnancy would lead to an increased risk for birth defects [57]. In contrast to several smaller studies, Charlton and colleagues found that the use of oral contraceptives during or before pregnancy had no association with the frequency of major birth defects. Unfortunately, they used these data to conclude that the inadvertent exposure of embryos and fetuses to EE2 would cause no harm to these individuals; lessons from DES have clearly shown how individuals exposed to synthetic estrogens during gestation can appear 'normal' at birth but develop diseases with a longer latency [10].

Although the long-term effects of developmental EE2 exposures on humans are uncertain, several controlled laboratory studies suggest that even low doses of EE2 can affect the developing rodent. In a large multi-generational study conducted by the National Toxicology Program, rats were orally exposed to a range of doses of EE2 over four generations [58]. The authors concluded that adverse effects were induced by developmental exposures, including decreased body weights, altered estrous cycles in the females, induction of male mammary gland hyperplasias and mineralization of renal tubules.

Many studies of other EDCs historically have used EE2 as a positive control for estrogenicity; it is considered a prototypical ER agonist [59]. Although these studies have revealed many effects of EE2, they have also shown that the effects of other xenoestrogens do not always replicate the effects observed after EE2 exposure. For instance, one study observed how a mixture of xenoestrogens in the Douro River affected gametogenesis in fish [60]. The effects of this xenoestrogen mixture were distinct from the effects of 100

ng/L EE2. Fish exposed to the mixture developed higher levels of ovarian follicle atresia compared to the EE2 exposed group. In studies from the Vandenberg lab, we have also observed striking differences between the effects of BPS and EE2 on a range of maternal behavior and brain endpoints in mice [61]. For example, F1 females exposed to BPS during early development spent significantly less time on the nest compared to untreated controls; this was not observed in EE2 treated females [48, 62]. Of course, differences in the relative doses of xenoestrogens and EE2 may be responsible for some of the differences observed, but it should not be discounted that various ER agonists may induce a range of variable biological effects that are not all reproduced by EE2.

Although there are a few studies examining the effects of developmental exposure to EE2, there is relatively little information about its effects at low doses, and in particular doses found in the environment (e.g. doses far below those prescribed to women). Because of its widespread use, EE2 is also commonly found as an environmental contaminant, particularly in waste water and drinking water [63, 64]. EE2 also has molecular features that distinguish it from the endogenous estrogen 17β-estradiol; for example, EE2 preferentially binds ERα over ERβ and never achieves maximal agonist activity via ERβ [65]. For these reasons, it may not serve as the optimal positive control for endocrine disrupting studies.

Critical Windows of Susceptibility

Developing organisms are highly susceptible to perturbations including exposures to EDCs [66]. There are periods of vulnerability throughout life, and during these periods, creatures are more sensitive to environmental disruptions. Many studies have showed that

adult disease outcomes are partially influenced by genetic factors, but a greater appreciation for the role of the environment, including chemical exposures, has recently been acknowledged [67].

In the 1990s, British scientist David Barker conducted a geographical study comparing infant mortality in babies with low birthweight in England and Wales [68]. These two regions of the UK had different socioeconomic statuses and Barker found that the region with babies that were nutritionally disadvantaged during *in utero* development were more likely to develop coronary heart disease later in life. An appreciation for the link between low birth weight and coronary heart disease developed from this study, supported by additional work in other populations [69].

Barker's work led to the idea that malnutrition during intrauterine development can lead to permanent changes in the offspring that can then give rise to adult diseases later in life. This was originally coined the Barker Hypothesis. The hypothesis gained popularity around the world and the idea of "developmental plasticity" intrigued the minds of research scientists from a range of scientific disciplines including endocrinology [70]. Over time, the hypothesis was transformed to the Fetal Origins of Health and Disease (FOHaD) or the Fetal Basis of Adult Disease (FeBAD). These concepts were further modified by a scientific society, which is now called the Developmental Origins of Health and Disease (DOHaD) society.

Gestation is a critical window of development, and proper growth and differentiation of cells, tissues and organs are critical to proper growth and ultimately to the health of the individual [71]. Each tissue type or organ has a key period of development which begins during gestation and can extend into the postnatal period. These periods of

development are susceptible to environmental insults. Thus, disruptions during vulnerable windows of development can induce future diseases.

Although embryonic and fetal development are obvious critical windows of development for virtually all organs of the body, other critical periods of sensitivity include pregnancy, puberty, and menopause. Animal experiments are useful for determining the windows of susceptibility for each target organ or tissue [72]. Rodents are particularly useful because their developmental periods are shorter, more easily observed, and environmental chemical exposures during these specific periods are better controlled compared to human epidemiological studies.

Mouse mammary gland development

The female mouse has five pairs of mammary glands positioned under the skin, consisting of three thoracic pairs and two inguinal pairs [73]. Once fully developed, each mammary fat pad contains a nipple and a lymph node. The lymph node offers a central landmark that can be used to evaluate the growth of the mammary gland throughout fetal and postnatal development.

The emergence of the mammary gland occurs at approximately embryonic day 10 (E10) as the epithelial anlagen forms over an underlying mesenchyme [74]. At E12, the epithelial bud develops layers and is more rounded; it remains a solid anlagen. Up until E13, the male mammary gland develops similarly to the female mammary gland. Then, the testes start producing androgens causing the rudimentary gland to detach from the epidermis in a manner dependent on androgen receptor, which is expressed in the mammary mesenchyme. This is why the male mammary gland lacks nipples [75, 76]. By

E14, in the female, the mammary rudiment is more bulb shaped and begins to invade the underlying mesenchyme. For the remainder of fetal development, the mammary anlagen will grow and branch, and will eventually develop a duct due to apoptosis of cells at the center of the anlagen at approximately E18 [77]. The mesenchyme will also differentiate into connective tissue containing collagen fibers and adipocytes.

By birth, the mammary gland is comprised of a rudimentary ductal tree [74] (see Figure 1.2). Few changes in the development of the ductal tree are observed in the first 3 weeks after birth, when the tree grows at approximately the same rate as the rest of the body. Just prior to puberty (approximately PND 21-24), the fat pad is comprised of a mixture of brown fat (multilocular adipocytes) and white adipose. The ductal tree is mainly posterior to the lymph node. With the onset of ovarian function and the production of estrogen, terminal end buds (TEBs) begin to appear at the ends of the mammary ducts [78] (Figure 1.2). TEBs are important for the rapid development of the ductal tree during prepubertal and pubertal stages. They are bulb like structures with epithelial cells at the "cap" that are highly proliferative, allowing the ducts to progress through the fat pad. As the mouse reaches the height of puberty (approximately PND32-35), the ductal tree will grow past the lymph node; more branching points are observed and more of the fat pad is filled.

By adulthood, the mammary ductal tree contains more secondary and tertiary branching, and the TEBs have regressed to form terminal ducts [79]. The ductal tree begins to develop lateral and alveolar buds in response to hormones produced throughout the estrous cycle (Figure 1.2). These alveolar buds are hollow epithelial structures involved in milk secretion. During pregnancy, the mammary gland epithelium undergoes a massive

phase of proliferation and differentiation in preparation for lactation [80]. In response to increased serum levels of progesterone and prolactin, the alveolar buds further differentiate into lobuloalveolar structures. One day before parturition, the lumena become filled with milk and lactation continues for about 3 weeks until pups are weaned naturally. At this point, the mammary gland experiences extensive cell death in a process termed involution [75]. The epithelial cells in the lobuloalveolar structures undergo apoptosis. By day 6 after involution, these structures are no longer present and the stroma and epithelium are rearranged. This process continues until the mammary gland returns to an appearance similar to a pre-pregnant mammary gland (Figure 1.2).

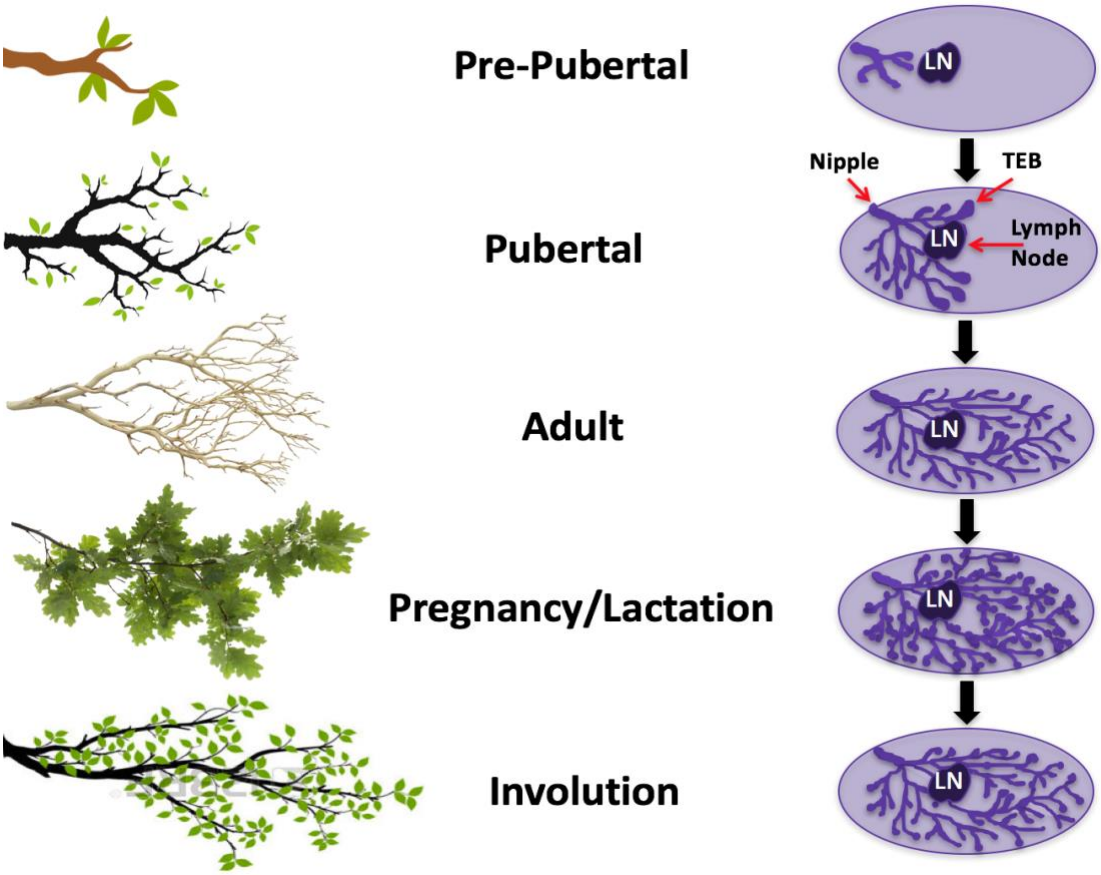

Figure 1.2. A schematic describing the development of the mouse mammary gland. A metaphor of a growing tree is used to describe the complexity of the epithelial structures from birth (at the top) through adulthood, pregnancy/lactation, and then post-lactation after the gland has undergone involution.

Effects of Hormones on the Mammary Gland

As described in the previous section, the mammary gland undergoes different phases of development at the embryonic, pre-pubertal, pubertal, pregnancy, lactation, and involution stages in a mammal's life. During these time periods, the mammary tissue is strongly influenced by hormones [75]; for this reason, scientists have hypothesized that synthetic chemicals with hormonal activities like BPS and EE_2 could disrupt mammary gland development and even lead to diseases like breast cancer [81].

For several decades, scientists have been working to identify which hormones and endocrine signals drive epithelial cell proliferation and gland differentiation [75]. During embryonic development, at approximately E10, 5 pairs of ectodermal placodes form along the skin. The epithelial and mesenchyme communicate (through parathyroid hormone related peptide), slowly increasing the size of the placodes. Throughout embryonic development, mesenchymal signals drive epithelial development. These mesenchymal cells express estrogen receptors (ER) and androgen receptors (AR) as well as other transcription factors and growth factors. At these embryonic stages, the epithelium does not typically express either hormone receptor. Thus, mammary gland responses to hormones are driven by mesenchymal-epithelial interactions.

During pubertal development, the growth of the mammary gland remains dependent on hormones [75]. Estrogen and progesterone are critical for various aspects of growth, and the absence of these hormones (or their receptors) impairs or restrains ductal development. Steroid receptor coactivators (SRC) also play a role in estrogen and progesterone signaling, and thus affect ductal growth.

The Jak2-Stat5 pathway, regulated by prolactin, promotes proliferation and differentiation of secretory mammary epithelium during pregnancy [75]. Peptides also control the development of the epithelial and stromal compartments. RANKL, an osteoclast differentiation factor, is important for alveolar production in the mammary gland; RANK and prolactin receptor pathways are crucial for cell proliferation. Lastly, cell cycle proteins like cyclinD1 and p27, which respectfully function to fully develop mammary tissue during pregnancy and to maintain cell proliferation rates and differentiation, both act antagonistically in the mammary epithelium.

The mammary gland responds to environmental factors

As an undergraduate working in the Vandenberg lab, my Honors thesis work determined that the mammary gland can be collected from male or female mice at pre-puberty, puberty, or in adults and can serve as a 'sentinel' tissue to distinguish animals with different developmental hormone exposures. Specifically, we found that the morphology of the mammary gland was different in animals raised in a commercial laboratory compared to animals raised in our laboratory, where we had taken efforts to limit their exposures to EDCs. This work was published in the journal *Environmental Health* [82].

Low Dose Effects of BPA and BPS on the Development of the Rodent Mammary Gland

Developmental exposures to BPA have been shown to alter mammary gland development in both mice and rats. In 2001, the first study to examine the effects of fetal

exposure to BPA revealed its effects on the developing mouse mammary gland [83]. This novel study reported disruptions to mammary gland development that were measured during neonatal, pubertal, and adult stages of mammary gland development. The study authors found that exposure to BPA altered the communication between the stroma and epithelium via alterations in DNA synthesis at all stages; an increase in terminal ends (TEs) was also observed in the 6-month old mice. This is important because these are one of the sites where carcinomas are thought to arise.

Follow-up studies have similarly reported decreases in the number of apoptotic cells in the TEBs of pubertal animals following perinatal exposure to BPA [84]. This is important because failure to undergo apoptosis in the center of a TEB could disrupt ductal patency, and could also lead to the retention of TEBs later into adulthood. Munoz-de-Toro and colleagues also found that a high number of epithelial cells were expressing progesterone receptor (PR), indicating that in BPA-exposed mice there is an increase is estrogen sensitivity of the mammary gland. There was also an increase in ductal branching in 4 month old BPA-exposed mice, consistent with increased expression of PR. Additional studies have revealed that BPA exposure during fetal development alters a number of aspects of mammary gland development including increased growth of the epithelial anlagen paired with decreased lumen formation, advanced fat pad maturation, and altered collagen deposition [77].

Similar concerns have been raised by studies of BPA exposed rats. In a recent study, exposure of female Wistar rats to low doses of BPA induced an increase in intraductal hyperplasias in adulthood [85, 86]. These studies and many others have provided consistent evidence that environmental estrogens, and BPA in particular, can profoundly affect

development and disease of the mammary gland. The effects of BPA manifest in a dose-dependent, exposure period-dependent, and age-dependent manner.

The male mammary gland has also been shown to be sensitive to estrogenic chemicals. The female and male mammary gland development is parallel through embryonic day 13, which is right before a testosterone surge from the male testes that induces a separation of the epithelial anlagen from the epidermis [87]. Perinatal exposure to BPA in male mice induces morphological alterations in adulthood and studies on the male mammary tissue can give insight on the most common human male breast disease, gynecomastia.

In a recent study, we evaluated growth and development of the mammary gland in females exposed to BPS or EE2 from gestational day 9 through postnatal day (PND) 21 . These animals were evaluated prior to puberty (PND21), during puberty (PND32-35) and in early adulthood (week 9). We found dose, stage, and chemical specific effects on mammary gland morphology, proliferation, and expression of hormone receptors. This work is in press at the journal *Reproductive Toxicology*.

Precocious Puberty

Puberty is a time when there are many changes happening in the body at the level of physiology, organ growth, and hormone signaling/action. The start of puberty is characterized by an elevation in hypothalamic gonadotropin releasing hormone (GnRH) [88] which activates the release of luteinizing hormone and follicle stimulating hormone. These hormones further induce the ovaries and testes to produce estrogen and testosterone.

Estrogen is a key factor in sexual differentiation. In females, the surge of estrogen is known to induce mammary gland growth at puberty [89].

Over the past few decades, the mean inception of thelarche (pubertal growth of breast tissue) and menarche (onset of the menstrual cycle at puberty) have both been decreasing. Girls are now entering pubertal development at the average age of 9.86 years compared to the 1990s, where the average age was 10.8 years [90]. Scientists have hypothesized that environmental EDC exposures, and in particular xenoestrogen exposures, can induce puberty, and therefore that the decreasing age of female puberty is due to increased exposures in human populations. Mueller and Heger show that BPA promotes early puberty by triggering *KiSS1*, which is critical for the onset of puberty, and that BPA also inhibits suppressing factors of puberty such as *Eap1* and *Yy1* [91]. The early onset of puberty has a number of downstream consequences; for example, precocious puberty has been shown to lead to a higher risk of developing breast cancer [21, 88].

The Two-Hit Model of Carcinogenesis, Extended to Hormone Responsiveness

The two-hit model of carcinogenesis was originally proposed in the 1970s by cancer researcher Alfred Knudson. Knudson found a link between the number of genetic mutations an individual was either born with or acquired in the Rb gene and the development of retinoblastoma [92]; individuals born with one mutation who acquired a second mutation later in life would develop a "hereditary" form of the disease. Alternatively, individuals that acquired two mutations after conception would develop a "spontaneous" form of the disease.

Cancer biologists have thought of other aspects of carcinogenesis as occurring in a two-step fashion, where some compounds can *initiate* the development of cancers, while other compounds can *promote* the development of cancers; some compounds can do both of these, and are considered *complete carcinogens* [93]. It has been thought that initiators must induce mutations, whereas chemicals that can induce cancers without causing mutations (such as saccharin, phenobarbitone, and hormones like 17β-estradiol) were characterized as cancer promoters.

In the field of endocrine disruption, numerous studies have taken the concept of "two hits" to evaluate how early life exposures to EDCs can influence the response of individuals to a secondary stressor encountered later in life. For example, mice exposed to EDCs during early development have enhanced responses to dust allergy mites, an environmental way of inducing asthma-like effects in rodents [94].

A few studies have evaluated the effects of gestational exposure to environmental chemicals and their influence on postnatal hormone challenges. One study found that early life BPA exposure altered the response of the mouse mammary gland to a low dose of estrogen during puberty in ovariectomized females [84]. A follow-up study compared this "two hit" response in two strains of mice, CD-1 and C57B16, and evaluated the mammary gland after perinatal exposure to BPA and its response to an estrogen challenge at puberty [95]. Both strains demonstrated altered responses of the mammary gland in response to a secondary estrogen exposure. Gomez et al. assessed the effects of the mammary gland in ovariectomized middle-aged rats given estrogen replacement therapy for three months following exposure to 0.5 or 50 μg BPA/kg/day during development [96]. This study showed that BPA-exposed rats developed mammary gland hyperplasia and benign cysts.

Although these rats were not producing endogenous estrogen, and BPA-exposed rats that were not challenged with estrogen were not examined, this study is consistent with others showing that early life BPA exposure sensitizes the rodent mammary gland to later life estrogen exposures.

Another series of studies evaluated prostate cancer risk in male Sprague-Dawley rats that were exposed to 0.1 to 5,000 µg BPA/kg early in life and then later in life given a hormonal challenge with testosterone and estradiol [97]. The adult treatment was meant to mimic hormone levels in older adult men. This study revealed that early life exposure to low doses of BPA alone had little effect on the prostate, but paired with a successive adult exposure to hormones resulted in prostatic intraepithelial neoplasia (PIN) and in some cases adenocarcinoma of the prostate [98].

A series of studies have evaluated the effect of developmental BPA exposure on the response of the mammary gland to chemical carcinogen challenges later in life. Overwhelmingly, these studies conclude that BPA increases the response of the mammary gland to carcinogenic chemicals [26]. In one example, cancer risk was evaluated in Sprague Dawley rats that were exposed to 25 or 250 µg BPA/kg BW during perinatal development and then administered DMBA, a chemical carcinogen, at puberty [99]. This two-hit experiment revealed an increase in tumor multiplicity and a decrease in tumor latency in mammary glands of animals exposed perinatally to BPA.

Finally, a recent study from the Vandenberg lab evaluated mice perinatally exposed to the BPA replacement chemical, BPS. Female mice were given a subsequent EE2 challenge a few days prior to puberty and effects of this "two hit" exposure were evaluated on the uterus and ovary [51]. Although BPS alone had few effects on either organ, BPS-

treated females provided an additional postnatal estrogen challenge displayed diminished ovarian development, and advanced uterine development, compared to estrogen-challenged females that were developmentally unexposed to BPS.

Hypothesis and Objectives

My Master's Thesis research aims to examine the role of early life exposures to estrogenic compounds and their impact on responses to estrogens encountered later in life, with a specific focus on the mammary gland. My work aims to expand on my undergraduate work to answer a new question: Does early life xenoestrogen exposure alter responses to hormonal challenges experienced later in life, e.g., at the onset of puberty, or when using contraceptive birth control, or hormone replacement therapy?

My thesis evaluates the effects of prenatal exposures to two xenoestrogens, BPS and EE2, on the response of the mammary gland to postnatal estrogenic challenges. I am specifically assessing whether a primary exposure to these xenoestrogens during gestation alters pubertal timing and mammary gland growth in response to a second hormone treatment either at puberty or at early or late adulthood.

We hypothesize that perinatal exposure to xenoestrogens will sensitize both male and female animals to estrogens, increasing the response of both males and females at puberty and adulthood. We anticipate that this heightened response will be seen in traditional measures of hormone action (organ weight), the timing of pubertal events (vaginal opening), and morphological parameters of the mammary gland.

Here, CD-1 mice were exposed to BPS, EE_2, or vehicle (Tocopherol Stripped Corn Oil) from pregnancy day 9 through lactational day 2. The pups were weaned at PND21,

and then given 10 days of estrogen supplementation (or vehicle) prior to puberty (starting at PND21), in early adulthood (starting at PND80), or in later adulthood (starting at PND260). Their mammary glands and other estrogen-sensitive tissues and endpoints were collected 24 hours after the last estrogen supplement. Completion of this project will allow us to better understand how environmentally relevant doses of synthetic estrogens disrupt development of the female and male mammary gland, and whether early life exposures sensitize individuals to hormonally active substances often used in later life for pharmaceutical purposes.

CHAPTER 2

DEVELOPMENTAL BPS EXPOSURE AND RESPONSE TO ESTROGEN AT PUBERTY

Introduction

Puberty is a developmental period that is controlled by, and dependent on, changes in hormone production and hormone action in both males and females. In humans, the hypothalamic-pituitary-gonadal (HPG) axis is active during fetal life, with production of Gonadotropin Releasing Hormone (GnRH) in the hypothalamus, luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the pituitary, and sex steroids (estrogen or testosterone) from the gonads [100]. The axis is active at birth, but serum concentrations of gonadotropins decrease during the first few months of age due to active suppression by inhibitory neurotransmitters, and only increase at the onset of puberty [101]. Although the physiological trigger for puberty is still unknown, a number of developmental landmarks are associated with this process including maturation of gametes, full maturation of the HPG axis, as well as the onset of adrenal androgen synthesis and secretion [100]. In human males, puberty is typically characterized based on the appearance of pubic hair, symmetrical testicular enlargement, and a linear growth spurt [101]. In human females, puberty is characterized by three separate morphological changes: *pubarche,* the appearance of pubic hair; *thelarche,* the onset of mammary development; and *menarche,* the onset of ovulation and menstrual bleeding [102]. The order in which these events occur is variable, and there is evidence that each event can be affected by genetic and environmental factors [103].

The mammary gland is an organ that is critical to reproductive success. Prior to puberty, the mammary gland is comprised of a quiescent rudimentary ductal tree. At the onset of puberty in female mice, growth of the mammary epithelium is induced under influence from ovarian and pituitary hormones [104-106]. Estrogen and growth hormone are responsible for the rapid development of the epithelial portion of the gland into the mammary fat pad. These hormones induce the formation of terminal end buds (TEBs), and increase secondary lateral branch formation [107]. TEBs are bulb-shaped structures that are responsible for the growth of the ducts; they have a single layer of epithelial cells, termed cap cells, at the outer layer which are highly proliferative and undifferentiated. These cells also produce cytokines to break down the connective tissue in the stroma. As the cap cells proliferate, they promote ductal elongation through the fat pad past the lymph node. Their daughter cells differentiate into luminal epithelial cells; cells at the outer edge of the newly formed duct are maintained whereas cells at the center of the duct undergo apoptosis to create a hollow duct, a process referred to as ductal patency [108]. Bifurcation of the ducts also occurs with the help of TEBs, which extends epithelium to all portions of the mammary stroma including the outer edge of the fat pad. Once this point of development is reached, the cap cells undergo apoptosis and differentiate into myoepithelial cells leading to the regression of TEBs into terminal ends. Thus, once the ducts have filled the fat pad, TEBs are no longer observed, leaving an adult stage mammary gland [109]. The adult mammary gland remains relatively unchanged until pregnancy, lactation and involution.

In the mouse, the male mammary gland is distinct from the female gland even at birth; most male mice lack nipples due to hormone action during gestation. During the

early embryonic period, growth and development of the mammary gland progresses similarly in males and females in the absence of steroid hormones. On embryonic day 14, sexual dimorphism of the gland occurs due to a surge in the male hormone, testosterone, produced by the testes. The binding of testosterone to androgen receptor (AR)-expressing cells in the mammary mesenchyme induces a condensation of these cells around the epithelial tissue, leading to the pinching off of this tissue and its separation from the epidermis [76, 109]. This leaves only a small mammary rudiment in the male, and the formation of the nipple never occurs. The absence of nipples has led most researchers to ignore the male mouse mammary gland although nipple retention is commonly used to evaluate chemicals for anti-androgenic properties [110]. It is otherwise unknown if the pubertal male mammary gland can respond to either endogenous or exogenous hormones, or even what morphological structures are 'normal' in the epithelial cells that remain.

It has been hypothesized that EDCs play a role in the secular trends of advanced pubertal timing in both male and females in the US and Europe in comparison to the mid-1990s [111]. Expert panelists concluded that females showed a trend towards earlier age of breast development and menarche but that there was not sufficient data to draw conclusions for males [112]. A more specific review on male pubertal development and EDCs concluded that limited data for males showed that prenatal exposure to environmental chemicals including hexachlorobenzene (HCB), DDE, and PCBs were associated with earlier pubertal onset in boys [113]. Epidemiological studies in the US, Europe, and China haven also shown that thelarche is occurring at younger ages over the past few decades [111].

Consistent with these secular trends, animal studies have shown that the HPG axis may be affected by developmental exposures to a range of estrogenic chemicals including DDT, 17β-estradiol, phytoestrogens, BPA, and estradiol benzoate, which induce earlier pubertal onset by stimulating GnRH secretion [111]. Estrogenic chemicals like BPA, genistein, resveratrol, zearalenone, and DES have been shown to accelerate vaginal opening in rats after prenatal exposures [114]. In contrast, phthalates, which are typically characterized by their ability to block synthesis of androgens, have been shown to interfere with preputial separation and testicular steroidogenesis in male rats, disrupting the onset of puberty [115]. A number of phthalates have been shown to induce nipple retention in male rodents [116-118].

There are only a few studies that have addressed the links between EDCs and pubertal timing. The Endocrine Society's second scientific statement on EDCs discusses how pubertal findings in both human and animal studies are equivocal and concludes that further studies must be conducted to gain a clearer understanding on how EDCs affect puberty [6]. In spite of important data gaps, however, the studies that are available do suggest a causal role for EDCs in abnormal pubertal development. Pubertal timing is important because it can have long term effects on the individual such as an increased risk for breast, ovarian, and testicular cancer, as well as metabolic syndrome, depression, anxiety, and other psychosocial hardships [119]. Thus, puberty is a measure that is critical to reproductive health and fitness.

Most studies evaluating the association between environmental chemical exposures and altered pubertal timing, at least in females, have focused on EDCs that mimic the actions of estrogen. One environmental chemical that has recently gained broad attention

by the public and environmental health scientists is bisphenol S (BPS), a chemical with estrogen receptor (ER) agonist properties [120]. This chemical analogue is used as a replacement for BPA in some consumer products. A systematic review of studies on BPS found that humans are exposed to BPS from consumer products including foodstuffs [121]. Non-food sources of BPS are also likely. One study collected indoor dust samples from the U.S., China, Japan, and Korea in various spaces including households, workspaces, laboratories, college dormitories, and floors [122]. They found the highest concentrations of BPS in the samples from Japan and the U.S. and suggest that humans may be exposed to BPS through dust inhalation and ingestion. Using estimates of dust inhalation and ingestion, the authors calculate that toddlers have the highest daily intake of BPS in all four countries evaluated, followed by children and adolescents. Another study, specifically examining BPS in paper products, concluded that a major source of exposure for the general population and occupationally exposed individuals is dermal contact with thermal receipt paper, with Japan and the U.S. being the two countries with the highest concentration in their paper products [35]. Another study that measured bisphenol analogues in surface waters found that India had the highest levels of BPS compared to other Asian countries; the authors speculate that this may be due to the production of BPS in India [123]. Finally, a recent study reported that BPS was detected in textiles and children's clothes, suggesting another non-oral route of exposure [124]. These studies collectively indicate that human exposures to BPS are likely increasing in many countries and there is a need for research to assess the potential health hazards that BPS may impose.

Although there are few studies examining the effects of BPS on estrogen-sensitive organs in mammals [121], work from our lab has demonstrated that the mammary gland is

affected by exposures during critical windows of development. Female mice exposed to low doses of BPS during pregnancy and lactation displayed abnormal mammary gland morphologies and disrupted nursing behaviors [49]. At the end of the lactational period, it was evident that the mammary glands of BPS-treated females were less developed than unexposed controls, with decreased volumes of epithelial tissue. Structures critical for milk secretion in the lactating mammary gland, called lobuloalveolar units, were diminished in the BPS treated mice. However, dams with abnormal mammary glands demonstrated an increase in time spent nursing and exhibited a nursing position that allows an increase in the amount of milk delivery to the pups; this result is consistent with a behavioral change (e.g., more time spent nursing) in response to a lactational deficit. In spite of these adaptive behaviors, BPS-exposed offspring were smaller and had delayed achievement of developmental landmarks like eye opening. This study demonstrated that the adult female mouse mammary gland is sensitive to BPS exposure during pregnancy and lactation.

We also evaluated mammary gland development in female offspring exposed to 2 or 200 µg BPS/kg/day or 0.01 or 1 µg EE2/kg/day during gestation and the perinatal period. Mammary tissue was collected at the pre-pubertal, pubertal, and early adult stages [125]. At pre-puberty, females perinatally exposed to BPS had increased TEB size and a higher area of the gland containing TEBs compared to controls. However, mammary epithelial cells in BPS-treated females did not display increased expression of proliferation markers prior to puberty. At puberty, neither perinatal exposure to BPS or EE2 had significant effects on mammary gland development. However, there were interesting effects of developmental BPS exposure observed early in adulthood. BPS-treated females displayed altered mammary gland morphology with increased ductal branching and alveolar buds,

increasing the density of all mammary epithelium. Surprisingly, females developmentally exposed to BPS also had retained TEBs in adulthood; this result was shocking because TEBs are expected to regress in adulthood. BPS and EE2 exposed mice also had an increased incidence of intraductal hyperplasia in the histopathology analysis conducted in early adulthood.

Finally, in a small pilot study, our lab evaluated the response of mice to an early life BPS exposure and a subsequent pre-pubertal estrogen challenge [51]. In this study, female CD-1 mice were exposed to 200 µg BPS/kg/day from gestational day 8 until postnatal day (PND) 19, followed by a three-day pre-pubertal estrogen challenge [1 µg ethinyl estradiol (EE2) /kg/day] from PND19 through PND21. The response of the uterus and ovary were evaluated on PND22. Females prenatally exposed to BPS, provided a postnatal estrogen challenge, demonstrated a significant increase in the uterine epithelial cell height compared to control females provided the same postnatal estrogen challenge. Furthermore, the control group provided an EE2 challenge developed an increased number of various ovarian follicles compared to control females not provided an EE2 challenge, consistent with an induction of ovarian function. In contrast, the perinatal BPS treated females had no significant ovarian response to an estrogen challenge.

Here, we evaluate the effects of perinatal xenoestrogen exposures on the response of the mammary gland and other hormone-sensitive endpoints to a postnatal estrogen challenge. We specifically focus on measures of pubertal onset in mice including the weight of estrogen-sensitive organs, anogenital distance, timing of vaginal opening, and morphology of the pubertal mammary gland. Our results indicate different sensitivity of

males and females to a pubertal estrogen challenge, as well as different responses of estrogen-sensitive endpoints.

Methods & Materials

Treatment of Mice with Test Chemicals

Timed pregnant female CD-1 mice (Charles River Laboratories, Raleigh, NC) were individually housed (until parturition) in polysulfone cages with food and water (in glass bottles) provided ad libitum. The animals were maintained in temperature and light controlled (12h light, 12h dark, lights on at 0800 h) conditions at the University of Massachusetts, Amherst Central Animal Facility. All experimental procedures were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

Mice were allocated to groups using statistical software to equalize body weight at the beginning of the dosing period. From pregnancy day 9 to lactational day 2, dams were orally dosed with BPS, EE2 or vehicle (Tocopherol Stripped Corn Oil) (Figure 2.1). The chemicals were diluted in corn oil at a concentration administration of 1 µg oil for every 1 g of body weight. The diluted solutions were designed to deliver 2, 200 or 2000 µg BPS/kg/day or 1 µg EE_2/kg/day. In total, the number of pregnant dams in each of the treatment groups was: control, n=11; 2 µg BPS/kg/day, n=10; 200 µg BPS/kg/day, n=12; 2000 µg BPS/kg/day, n=10; 1 µg EE2/kg/day, n=10. Dams were trained to drink oil from the tip of a pipette, allowing chemicals to be administered orally while avoiding the overt stress of gavage. Doses were adjusted daily for body weight. Dams delivered naturally (birth designated lactational day 0 [LD0]). Litters were not culled.

At PND21, pups were moved to new cages, separated by sex, and weaned. Two females and two males from each litter were selected at random. One pup of each sex was assigned

to receive vehicle, and one was assigned to receive an estrogen challenge (Figure 2.1). Each pup orally dosed via a pipet with either vehicle (Tocopherol Stripped Corn Oil) or 1 µg EE2/kg/day. Doses were adjusted daily for body weight. The females were checked for vaginal opening all ten days of dosing.

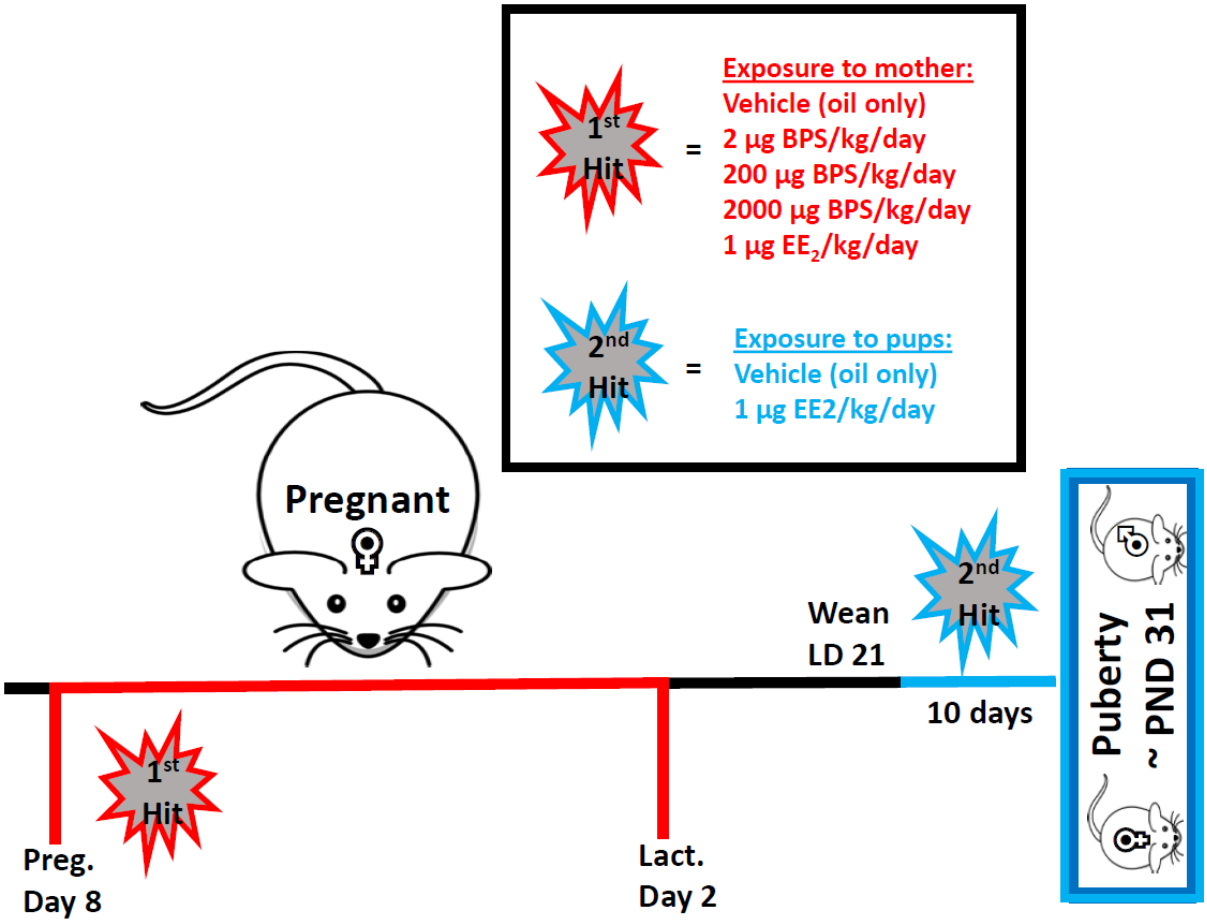

Figure 2.1. Experimental design schematic. Here, first-hit exposure is a developmental exposure from pregnancy day 8 through lactational day 2 (administered to the mother) and the second-hit exposure (estrogen challenge) is 10 days prior to puberty (administered to the pup). Both male and female pups were evaluated.

Necropsies and Dissections

Females

On PND31, female offspring were euthanized by CO_2 inhalation followed by decapitation. Trunk blood was collected and serum was isolated. A vaginal smear was collected to verify estrus cycle stage, and anogenital distance was measured with calipers. The left and right fourth inguinal mammary glands were isolated using standard dissection methods. The right mammary gland was spread on a positively charged slide and fixed in neutral buffered formalin for whole mount processing. The left mammary gland was drop-fixed in neutral buffered formalin for later paraffin embedding and histological analysis. The pectoral/thoracic mammary tissue was also collected and snap frozen for RNA. The uterus was weighed using an analytical balance. One horn of the uterus was fixed in neutral buffered formalin, while the other uterine horn was snap frozen for RNA.

Males

On PND31, male offspring were euthanized by CO_2 inhalation followed by decapitation. Trunk blood was collected for serum isolation, and anogenital distance was measured using calipers. The left and right fourth inguinal mammary glands were isolated using standard dissection methods. Both mammary glands were spread on a positively charged clear slide and fixed in neutral buffered formalin for whole mount processing. The seminal vesicles were weighed using an analytical balance.

Carmine Alum Whole Mount Staining

After 24-48 hours of fixation at room temperature, whole mount mammary glands were washed with phosphate buffered saline and stored in 70% ethanol. For staining, whole mounts were dehydrated through a series of alcohols and defatted with toluene. Next, the glands were stained overnight with carmine, dehydrated through a series of alcohols and xylene, and then placed in k-pax heat bags with methyl salicylate, an optically beneficial preservative agent.

Whole Mount Morphometric Analysis

Females

Whole mounts mammary glands from female offspring were imaged using a Zeiss Axio Imager dissection microscope. Using Zen Pro software, the whole mounts are quantitatively analyzed using methods developed previously [83, 126]. Specific measurements included the area subtended by the ducts (ductal area), the growth of the longest duct from the center of the lymph node (ductal extension), the total number of terminal end buds (TEBs, defined as bulb-shaped structures ≥ 0.03 mm^2), and area of TEBs.

Males

Male whole mount mammary glands were imaged using the Zeiss Axio Imager dissection microscope. Using Zen Pro software, the whole mounts were analyzed to quantify number of branching points, number of ductal trees, ductal area, ductal extension, number of TEBs, and the area of TEBs.

Embedding/Sectioning of Fixed Mammary Gland Tissue

Following fixation, mammary glands were washed and processed through a series of graded alcohols followed by vacuum infiltration of paraffin. After tissues were embedded in paraffin, the blocks were trimmed and stored at -20°C. Using a rotary microtome, mammary glands were sectioned at a thickness of 5μm, floated on a hot water bath and collected on positively charged glass slides.

Histological Staining

Sections were processed through a series of xylenes to deparaffinize the tissues, followed by a graded alcohol series (100%, 95%, 70%) to rehydrate the tissue. Samples were stained with hematoxylin and eosin (approx. 1 min for hematoxylin, 4-5 min for eosin) and dehydrated through an alcohol series and xylene. Samples were coverslipped with permount solution. Samples were evaluated using a Zeiss Oberserver.Z1 inverted microscope and 20x and 40x objectives. Images were collected with a Zeiss high resolution color camera.

Vaginal Smear Staining

At time of necropsy, a vaginal smear was collected on a glass slide. Smears were rapidly fixed in formalin and processed through a graded series of alcohols. Samples were stained with hematoxylin and eosin, dehydrated, and coverslipped with permount. Smears were viewed with a Zeiss Oberserver.Z1 microscope and scored as one of four estrus stages.

qPCR

Total RNA from the mammary gland tissue from the pubertal female mice was extracted using Trizol reagent and a BeadBug microtube homogenizer (Sigma Aldrich, St. Louis, MO) according to the manufacturer's instructions. RNA concentration and purity were quantified by UV spectrophotometry (Nanodrop 1000; Thermo Scientific).

One microgram of RNA from each sample was reverse transcribed to cDNA using reverse transcriptase (Applied Biosystems, Inc). The FastStart Universal SYBR Green Master kit (Roche Diagnostics Corporation, Indianapolis, IN) was used for the qPCRs along with 1 μL of cDNA and 300 nM forward and 300 nM reverse primers for each target gene. β-actin was used as a housekeeping gene. Every sample was run in triplicate for each gene target. The thermal profile was as follows: 10 minutes at 95℃; 40 cycles of 15 seconds at 95℃, 30 seconds at 60℃, and 15 seconds at 72℃; a melting-curve analysis was conducted to identify nonspecific products. Relative quantification was determined using the ΔΔCt method to correct for differences in β-actin [127]. Genes selected for evaluation are markers of estrogen action including Esr1 and PgrR.

Statistical Analyses

SPSS v24 was used for all statistical analyses. All data were collected by experimenters blind to treatment group. For all data collected from dams, one-way ANOVA followed by Fisher's LSD posthoc was used to assess differences in pregnancy outcomes. For all normally distributed data collected from pups, two-way ANOVA (with prenatal and postnatal treatments representing independent variables) followed by Fisher's LSD posthoc were used to assess the differences in morphological features between treatment

groups. Categorical data were evaluated with Chi Square tests. Results were considered significant at $p < 0.05$. All data presented in graphs represent mean ± SEM.

Results

Pregnancy outcomes in BPS- and EE2-treated dams

Female mice were weighed throughout pregnancy to adjust daily doses of vehicle, BPS or EE2. On the day prior to parturition, there were no differences in body weight between pregnant females in the five treatment groups (Figure 2.2A). There were also no significant differences in the length of gestation between treatment groups (Figure 2.2B) or the sex ratios of pups in the litter (Figure 2.2C).

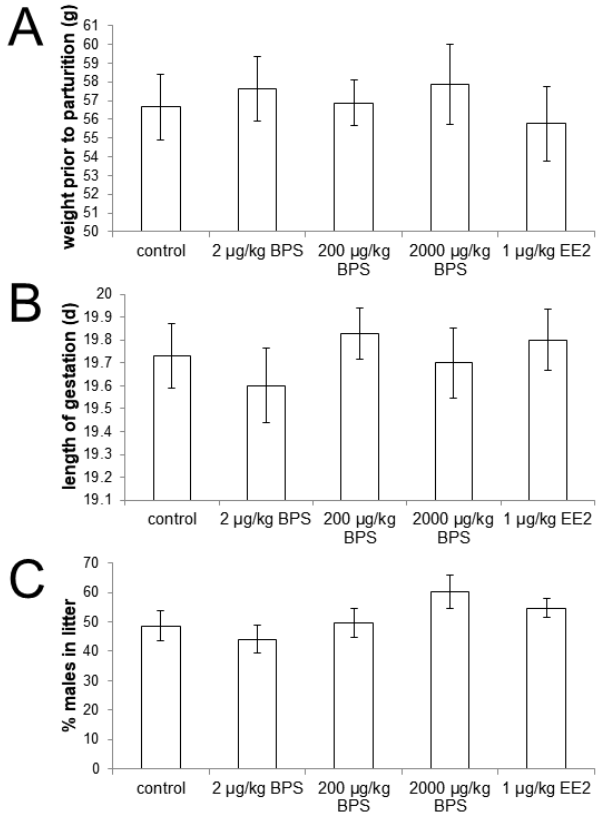

Figure 2.2. Pregnancy outcomes in xenoestrogen-exposed dams. A) There were no differences in body weight between treatment groups in dams one day prior to parturition. B) No differences in length of gestation were observed between treatment groups C) No significant differences in sex ratios of pups in litters were observed after BPS or EE2 exposure.

Average litter size and number of pups surviving to weaning were also not affected by treatment (Figure 2.3A,B), suggesting that the perinatal BPS and EE2 treatments were not overtly toxic to the pups. Although their frequency was not statistically significant compared to control litters, deformed pups were only observed in the highest BPS group (2 of 10 litters) and the EE2 group (1 of 10 litters). These pups appeared normal at birth but failed to develop properly, with stunted growth, improper appearance of fur, and delayed eye opening (Figure 2.3C). Prior studies from our lab have shown that BPS, but not EE2, delays eye opening at PND14. Here, a similar trend was observed, although these delays were not statistically significant (Figure 2.3D).

Prenatal xenoestrogen exposure alters anogenital index in males

Anogenital distance [AGD] (and anogenital index [AGI], which accounts for differences in body weight), is a standard indicator of prenatal hormone action. AGD and AGI are larger in males and smaller in females (Figure 2.4A). Prenatal exposure to estrogenic compounds or antiandrogenic compounds can diminish AGD/AGI in exposed male offspring; postnatal exposures are not expected to have these effects. 2-way ANOVA revealed a significant effect of prenatal exposure on AGI in male offspring ($p<0.05$) although posthoc comparisons did not reveal significant effects of any particular treatment group. There was no effect of postnatal exposures, but a significant interaction between prenatal and postnatal exposures was observed ($p<0.05$). These results suggest that prenatal xenoestrogen exposures influenced how AGI was affected by the postnatal EE2 challenge (Figure 2.4B).

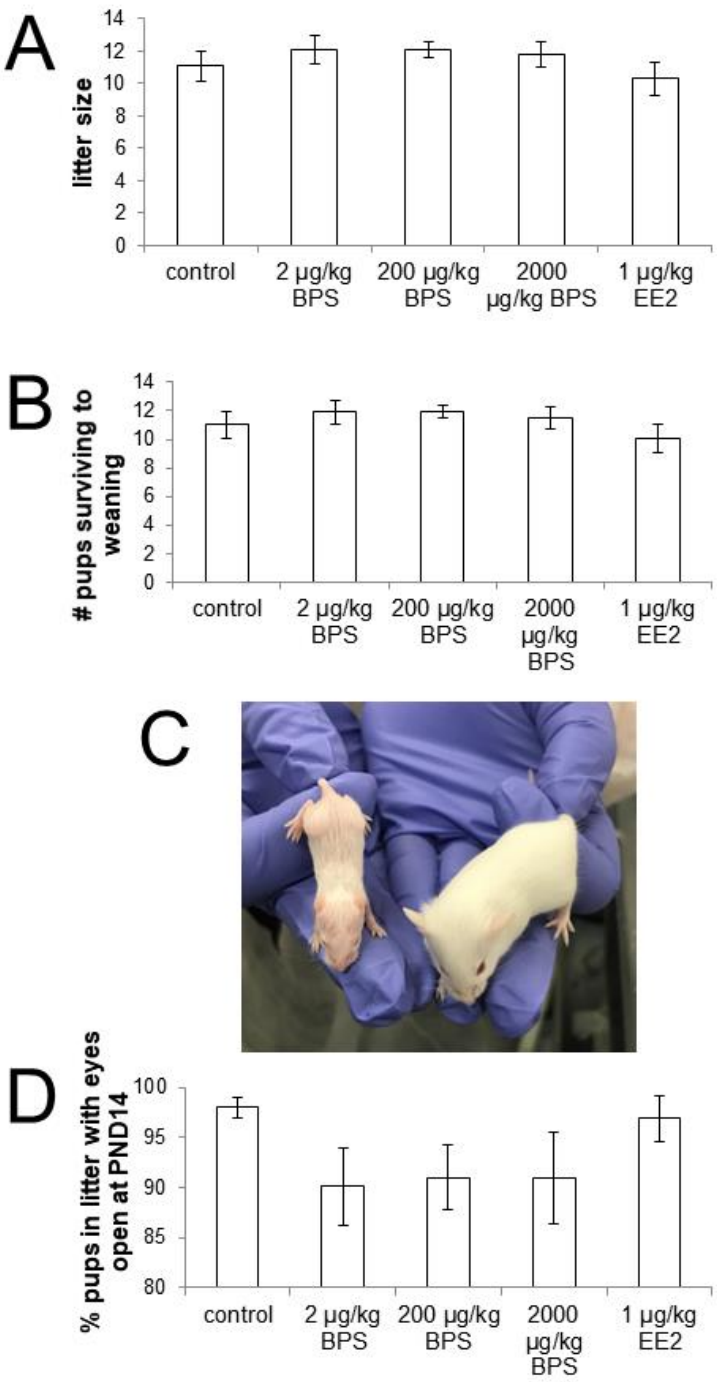

Figure 2.3. No overt toxicity was observed in litters from any treatment. A,B) Litter size and number of pups that survived until weaning were unaffected by treatment. C) Deformed pups were observed in the high BPS group (2/10 litters) and EE2 group (1/10 litters); this image shows improper appearance of fur and smaller body size in affected pups. D) Although not significant, delays in pup eye opening of at PND 14 were observed in the BPS treatment groups.

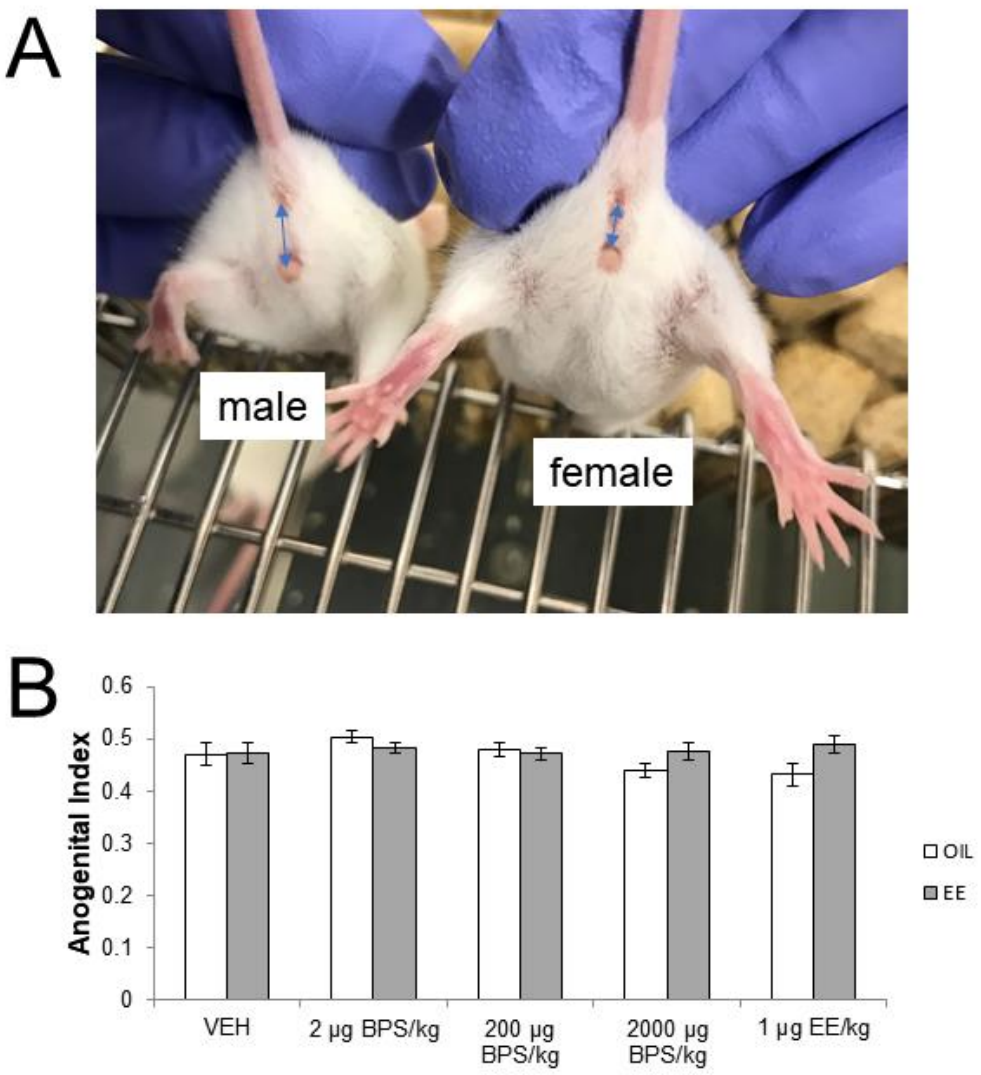

Figure 2.4. Anogenital distance (AGD/AGI) observations in males. A) AGD is normally longer in males and shorter in females. B) AGI, which accounts for differences in body weight, is another tool to evaluate AGD. There were significant effects of prenatal exposure on AGI in male offspring as well as a significant interaction between prenatal and postnatal exposures (2-way ANOVA, $p<0.05$).

Postnatal estrogen challenge alters seminal vesicle weight in males

We next measured the weight of the seminal vesicles, an estrogen- and androgen-sensitive organ that responds to postnatal hormone exposures. 2-way ANOVA revealed a significant effect of postnatal, but not prenatal, treatment ($p<0.05$). There was also a significant interaction between prenatal and postnatal treatment ($p<0.05$). As expected, in the control groups, the postnatal estrogen challenge significantly decreased seminal vesicle weight. In contrast, mice prenatally exposed to BPS or EE2 were non-responsive to the EE2 challenge (Figure 2.5). These results are consistent with a developmental xenoestrogen exposure that masks the effect of a postnatal estrogen challenge.

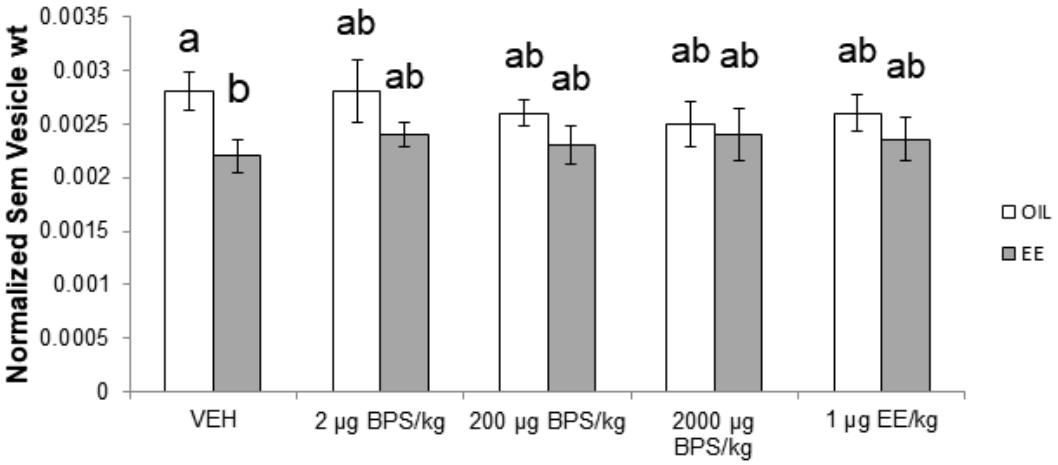

Figure 2.5. A postnatal estrogen challenge alters seminal vesicle weight in control males. Seminal vesicle weight (normalized to differences in body weight) decreases in response to a postnatal estrogen challenge in controls. Prenatal xenoestrogen exposure appears to mask this effect. Different letters indicate significant differences between groups ($p<0.05$, Fisher's posthoc after significant 2-way ANOVA).

Characterization of the male mammary gland at puberty

Previous studies have not evaluated or characterized the male mouse mammary gland at the pubertal stage. At PND31, the appearance of the male gland was similar to a

female mammary gland at the beginning of puberty (PND21-24, see Figure 2.6). Although the male epithelial tree was smaller in area than the female mammary tree, the male exhibited similar amounts of lateral branching. Surprisingly, a small number of TEBs were observed in the male mammary gland at PND31. The presence of these structures is interesting because their purpose is to advance the growth of the mammary epithelium through the fat pad, yet prior studies of the adult male mouse mammary gland have revealed very small ductal trees [87].

Consistent with other unpublished observations made in the lab (Pokharel, Kolla and Vandenberg, in preparation), there was a clear asymmetry in the two male mammary glands evaluated. In control males at PND31, the left gland was typically larger than the right gland. There was also more branching and an increased number of TEBs in the left compared to the right mammary glands.

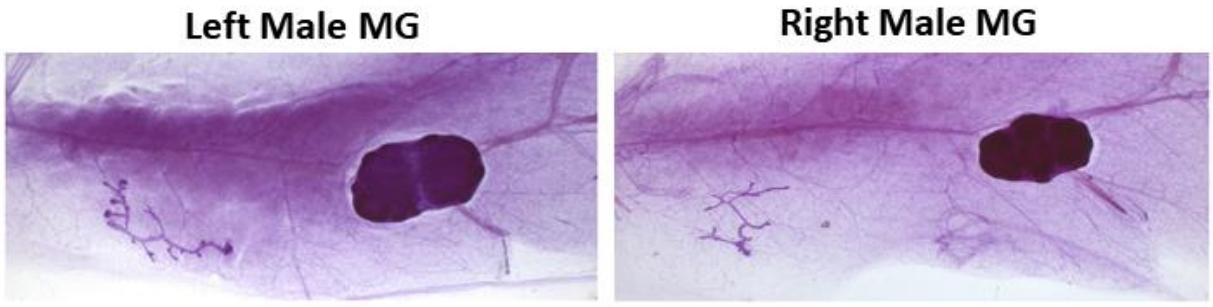

Figure 2.6. Male mammary gland at puberty. Images of typical left and right male mammary glands at PND31. There is an asymmetry of these glands, where the left is larger than the right.

Mammary gland morphology in pubertal males is disrupted by both prenatal xenoestrogen exposure and a postnatal EE2 challenge

Male mammary gland whole mounts were analyzed using quantitative volumetric morphometric methods. In the right mammary gland, 2-way ANOVA revealed overall significant effects on ductal area, number of branching points, number of TEBs, and total TEB area ($p<0.001$).

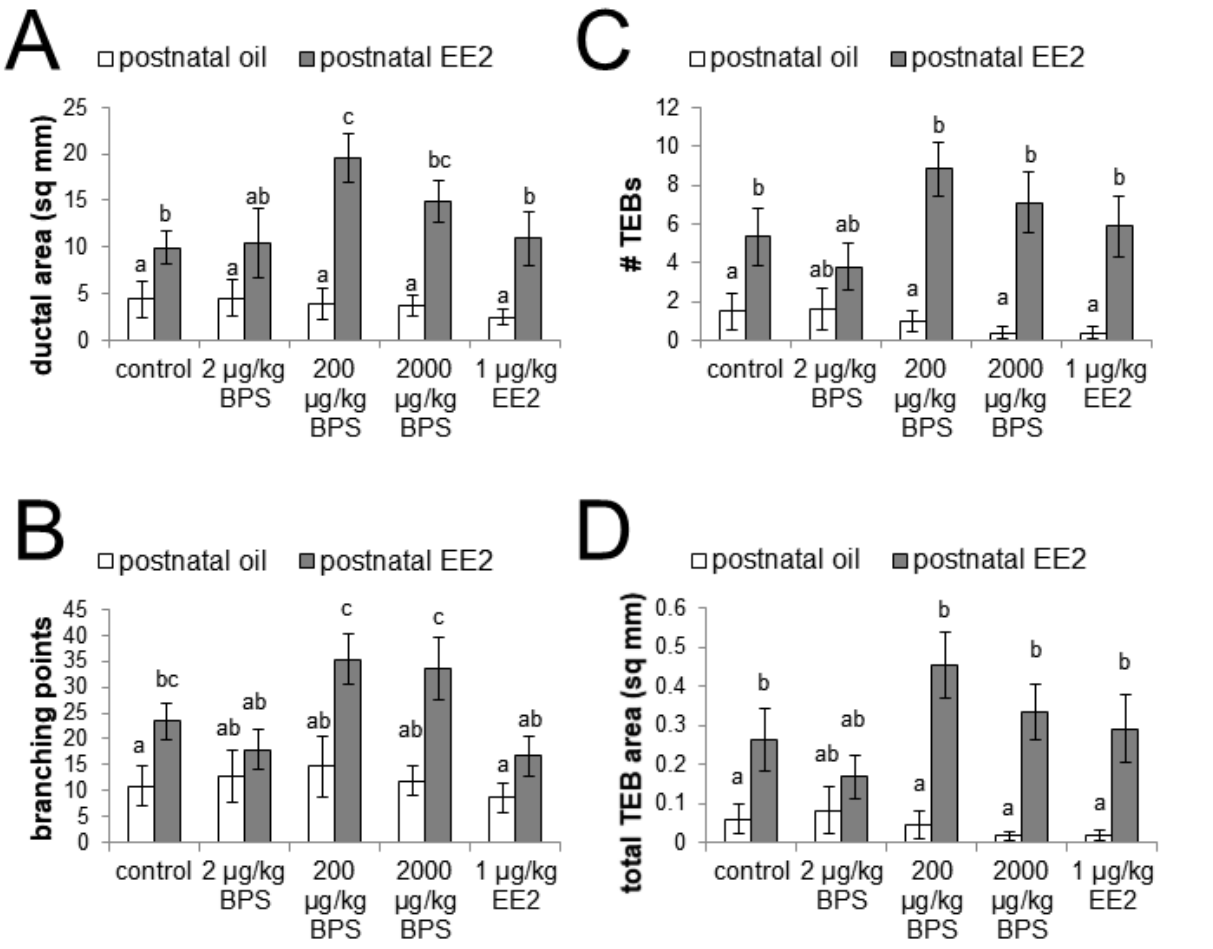

Figure 2.7. A postnatal estrogen challenge induces growth and development in the right mammary gland of males dependent on prenatal treatment. A) Area of the ductal tree; B) number of branching points; C) number of TEBs; D) total area of TEBs. Different letters indicate significant differences between groups ($p<0.05$, Fisher's posthoc after significant 2-way ANOVA).

Significant effects were observed for postnatal and prenatal treatment ($p<0.01$) and there was a significant interaction between postnatal and prenatal treatment ($p<0.05$). Graphing these growth parameters revealed that the postnatal EE2 challenge significantly increased growth parameters in males from the control group, as well as males prenatally exposed to 200 or 2,000 µg BPS/kg/day or 1 µg EE2/kg/day. In contrast, males prenatally exposed to 2 µg BPS/kg/day were non-responsive to the postnatal EE2 challenge (Figure 2.7). Representative images for the ten treatment groups are provided in Figure 2.8.

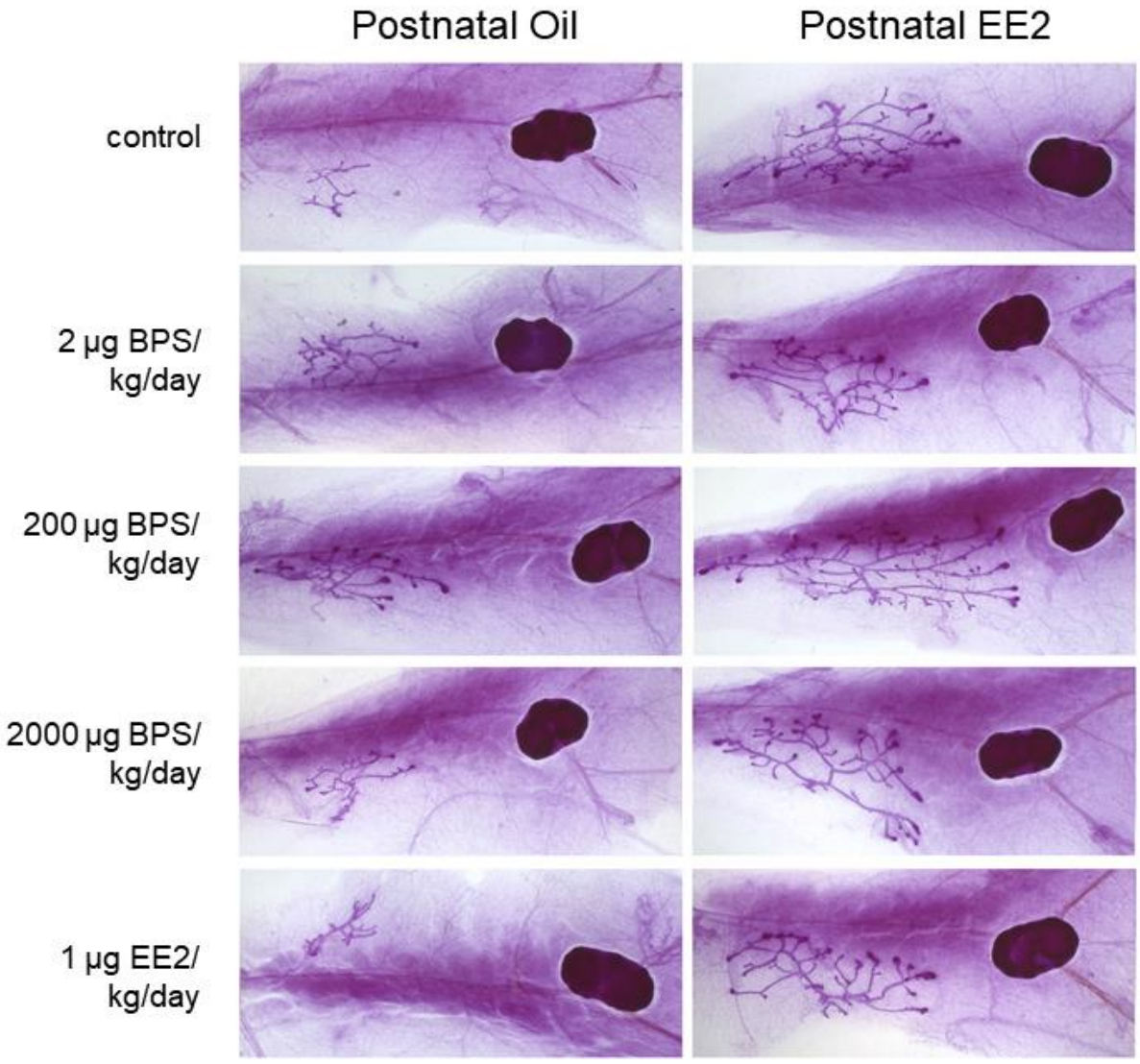

Figure 2.8. Right male mammary gland morphometric observations.

In the left mammary gland, fewer parameters were altered by either prenatal xenoestrogen treatment or the postnatal estrogen challenge. The overall ANOVA was significant for TEB parameters (number, total area) but not ductal area or branching points. For TEB parameters, there were significant effects of postnatal, but not prenatal treatment ($p<0.01$) although there was an interaction between the treatment groups. Graphing these parameters revealed that the postnatal EE2 challenge did not influence ductal area or branching points in controls, suggesting that the left mammary gland is not responsive to postnatal estrogen exposures. In contrast, animals prenatally exposed to the higher doses of BPS did respond to the EE2 postnatal challenge, with modestly larger areas and more branching points. Although EE2 increased the number and total area of TEBs in control males, these increases were not significant. In contrast, males prenatally exposed to 200 or 2000 μg BPS/kg/day or 1 μg EE2/kg/day were very responsive to the postnatal EE2 challenge in the TEB parameters measured (Figure 2.9). Representative images are presented in Figure 2.10.

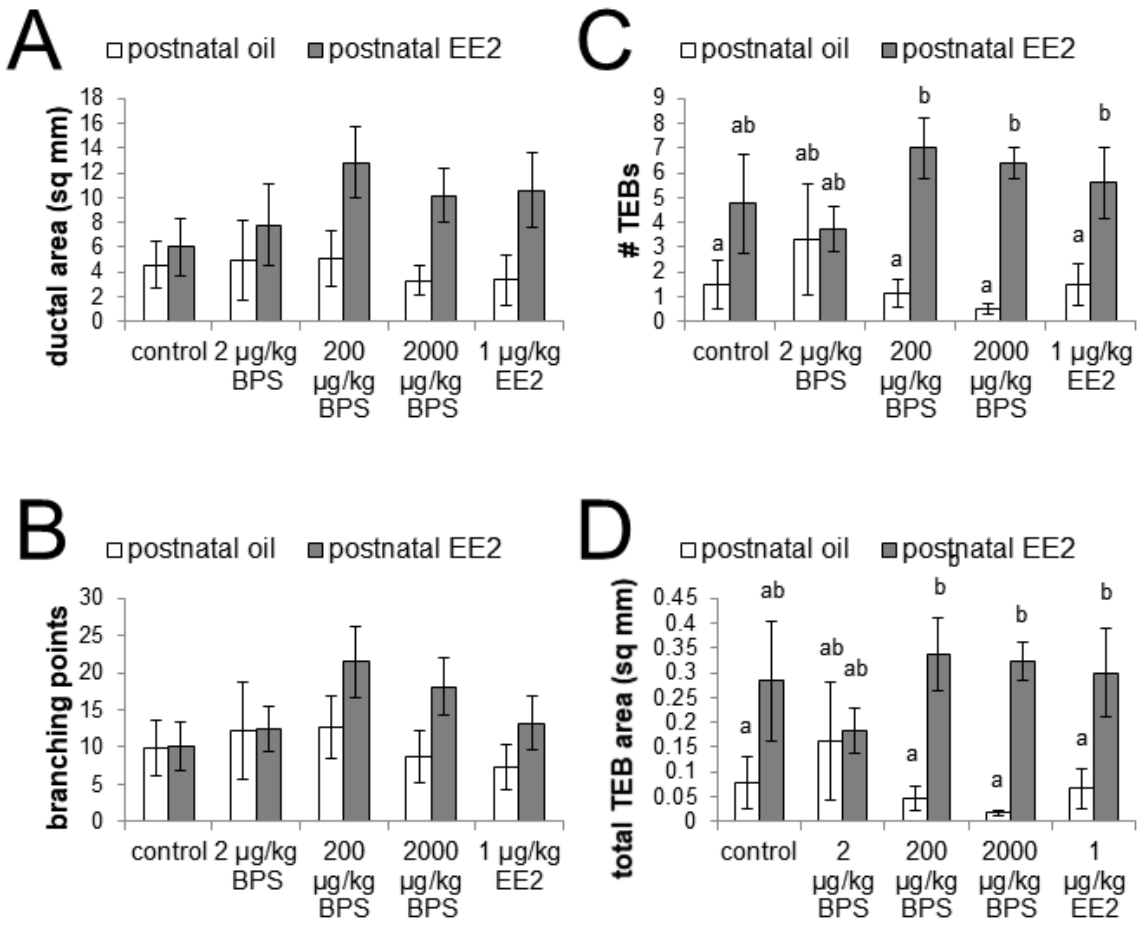

Figure 2.9. A postnatal estrogen challenge induces growth and development in TEB parameters of the left mammary gland of males dependent on prenatal treatment. A) Area of the ductal tree; B) number of branching points; C) number of TEBs; D) total area of TEBs. Different letters indicate significant differences between groups ($p<0.05$, Fisher's posthoc after significant 2-way ANOVA).

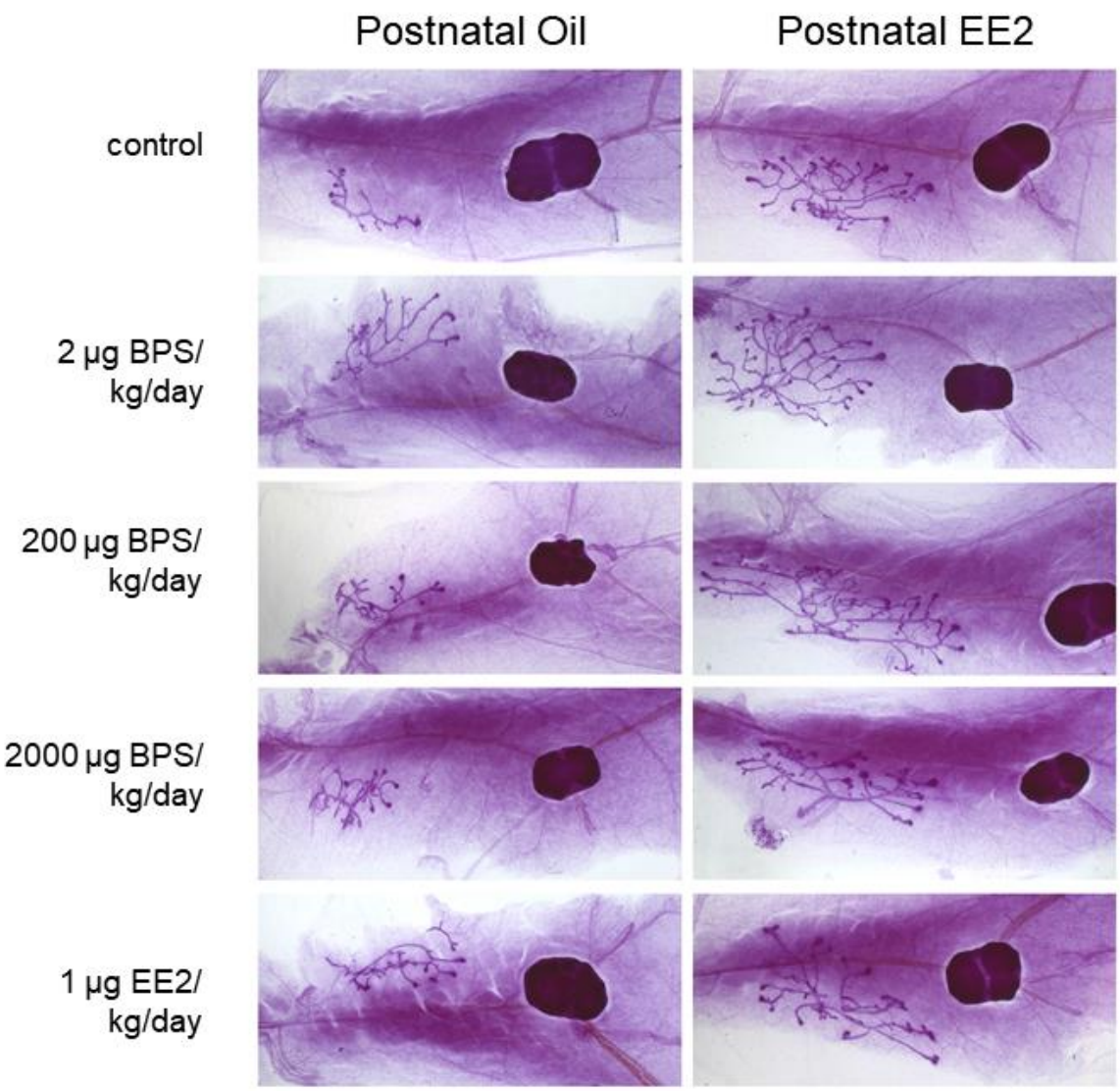

Figure 2.10. Left male mammary gland morphometric observations.

Neither prenatal xenoestrogen exposure nor a postnatal estrogen challenge alters anogenital index in females

In contrast to the effects of chemicals on developing males, AGD and AGI are typically not affected in female rodents exposed to estrogenic chemicals during gestation. 2-way ANOVA revealed no effect of either prenatal xenoestrogen exposure or the postnatal EE2 challenge on AGI in female offspring (Figure 2.11).

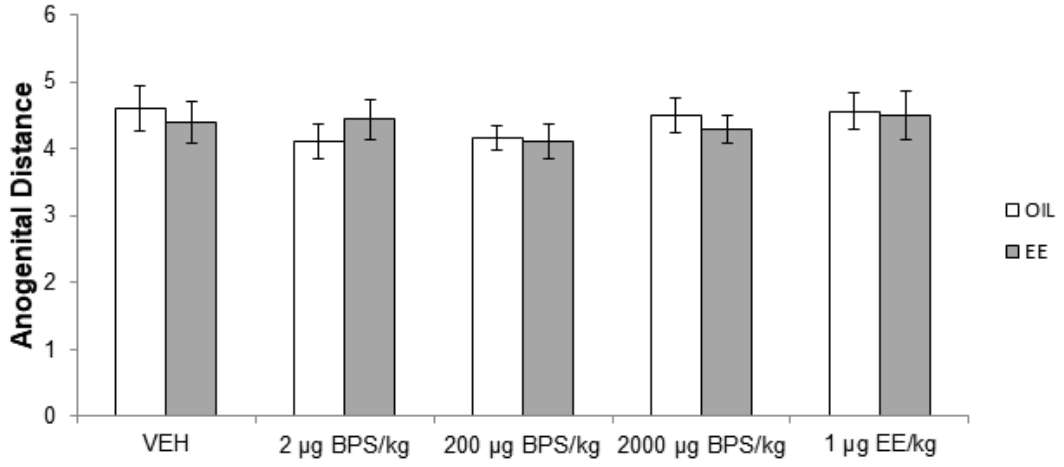

Figure 2.11. No effect of prenatal xenoestrogens or a postnatal estrogen challenge on AGD/AGI in females. Neither prenatal xenoestrogen exposure or postnatal estrogen exposure resulted in altered AGD in females. AGI was also not affected (not shown).

A postnatal estrogen challenge may advance the timing of ovarian puberty in control, but not xenoestrogen-exposed, females

In the mouse, ovarian puberty is typically evaluated by the timing of vaginal opening and/or the timing of first estrus. In control females, a postnatal EE2 challenge decreased the time to vaginal opening (from PND 27.6 ± 1.1 to PND 25.2 ± 1.0), although

this difference was not statistically significant. No changes in the timing of vaginal opening were observed in females perinatally exposed to BPS or EE2 (Figure 2.12).

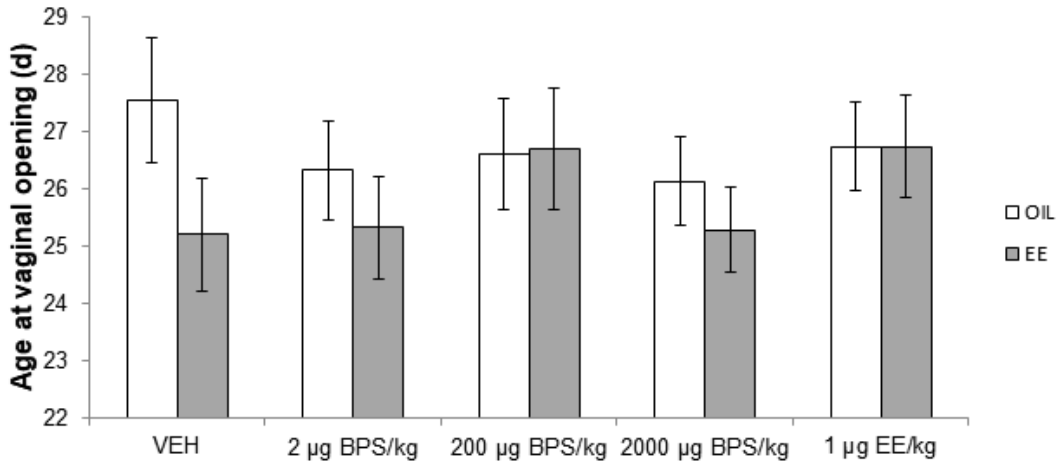

Figure 2.12. Timing of vaginal opening may be advanced by a postnatal estrogen challenge. Control females given a postnatal estrogen challenge had vaginal opening approximately 2 days earlier than unchallenged control females. This decline was not statistically significant. Females developmentally exposed to BPS or EE2 did not alter age at vaginal opening.

Neither prenatal xenoestrogen exposure nor a postnatal estrogen challenge altered uterine weight in females

In contrast to the effects of chemicals on weight of the seminal vesicle in exposed males, uterine weight was not affected by either prenatal xenoestrogen exposure or the postnatal EE2 challenge in female offspring (Figure 2.13).

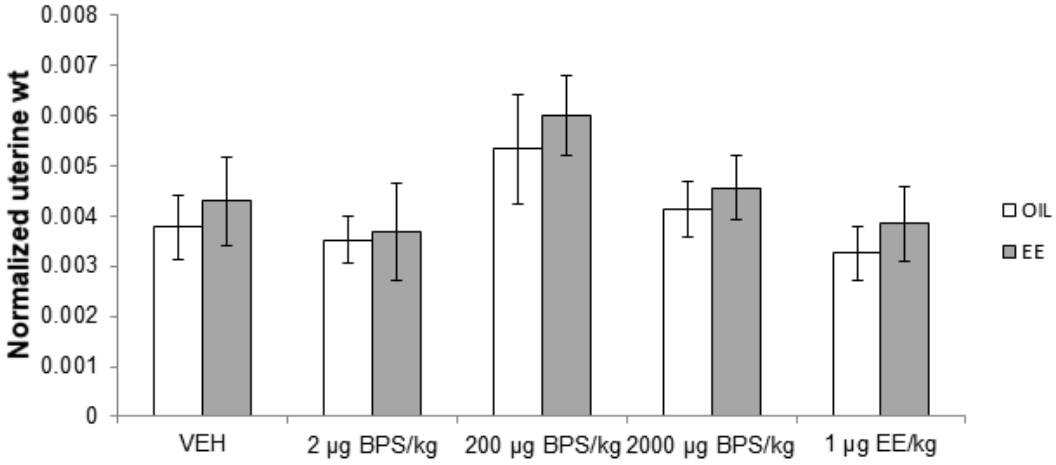

Figure 2.13. Normalized uterine weight was unaffected by developmental xenoestrogen or a postnatal estrogen challenge. Neither prenatal BPS or EE2 treatment or postnatal estrogen challenge altered uterine weight in females.

Female mammary gland growth parameters are not significantly affected by a pubertal estrogen challenge

Female mammary gland whole mounts were analyzed using quantitative volumetric morphometric measures. Measures of epithelial growth including ductal area (Figure 2.14A) revealed no significant differences based on either the prenatal xenoestrogen treatment or the postnatal estrogen challenge. Similarly, parameters related to the number and size of TEBs revealed no effects of either prenatal or postnatal treatment (Figure 2.14B). Representative images are presented in Figure 2.15.

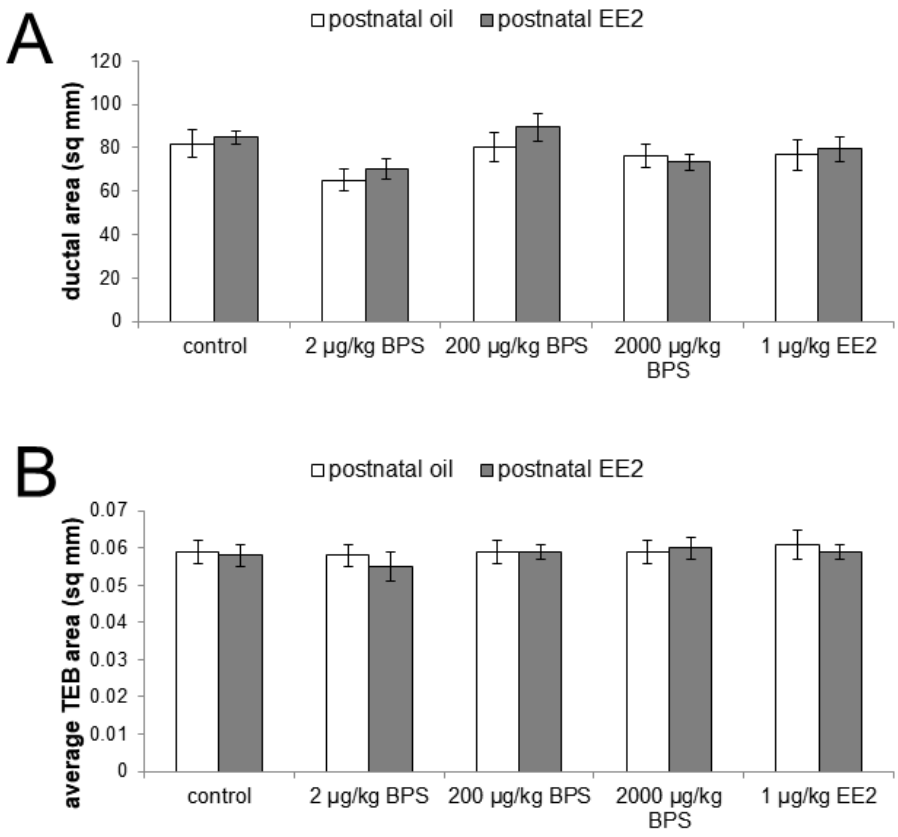

Figure 2.14. Pubertal female mammary gland morphometrics were not altered by treatment. A) Ductal area revealed no significant differences with developmental xenoestrogen or postnatal estrogen exposures. B) TEB related parameters also remained unaffected by either prenatal or postnatal treatment.

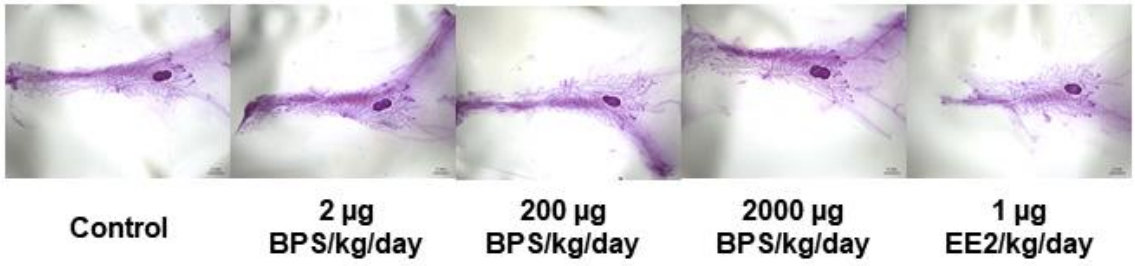

Figure 2.15. Female mammary gland morphometric observations.

Discussion

This study evaluated the effects of perinatal xenoestrogen exposures on female and male CD-1 mice at puberty. We were specifically interested in evaluating whether exposures to BPS or EE2 during early development would alter the response of animals to a postnatal estrogen challenge starting just prior to the onset of puberty. We examined mammary tissue in both males and females, as well as other estrogen-sensitive endpoints including AGD/AGI, weight of reproductive organs, and timing of vaginal opening.

We observed some effects of BPS on hormone-dependent endpoints, but the most interesting effects were only observed in BPS-treated animals after the pubertal EE2 challenge. With this combined "two hit" exposure paradigm, effects were observed on a range of endpoints including male AGD/AGI, male reproductive organ weight, and the timing of vaginal opening in females. Our results also revealed significant effects of this "two hit" exposure on pubertal male, but not female, mouse mammary gland development. Importantly, these effects were observed in the absence of overt reproductive toxicity. Our study revealed no effects of BPS or EE2 on maternal parameters including weight gain, length of gestation, number of pups born, litter sex ratio, or number of pups surviving to weaning. We did, however, observe modest changes in growth and developmental outcomes like delayed eye opening in the high BPS group, similar to what has been previously observed in BPS exposed litters [49]. Pups with stunted growth, which became obvious during postnatal development, were only seen in the high BPS and EE2 treated groups, although their incidence was not statistically increased compared to controls.

To the best of our knowledge, the male mouse mammary gland has not been characterized or studied at puberty. We found that the male mammary gland has an

appearance similar to the female gland at the onset of puberty (approximately PND 21-24). The gland is small – typically with a ductal area of only 3-7 mm^2 and 8-14 branching points. Surprisingly, at PND31, the control male pubertal gland does contain TEBs (normal range of 1-3 per gland). Our study revealed that the male mammary gland is sensitive to both perinatal xenoestrogen exposures and pubertal estrogen challenges. Even more interesting was the asymmetry that was observed in the male gland; there were distinct morphologies that were seen in the left versus the right glands. We also found no effect of a postnatal EE2 exposure on controls in the left gland, although, a prenatal exposure to some doses of BPS enhanced the growth of the left-sided gland. In contrast, the right mammary gland demonstrated sensitivity to postnatal estrogen because we observed that a postnatal EE2 challenge significantly increases the growth of the mammary gland on this side (Figure 2.16).

The modest effects of the postnatal estrogen challenge on the timing of vaginal opening are in contrast to the lack of effects of the postnatal estrogen challenge on mammary parameters. We speculate that this could suggest that thelarche and menarche are not synchronized in the pubertal mouse; in the human, there is evidence that these events occur separately and can be differently affected by environmental factors. In a recent U.S. cross-sectional study, the mean age of thelarche in girls was calculated at 9.7 years, whereas the mean age of menarche was 12.8 years [128]. Importantly, the authors found that thelarche was advanced by about 1.4 years over two decades, whereas the mean age of menarche had remained unaffected over the past 20 years. Another research team evaluated the age of thelarche and menarche in a Copenhagen puberty study, using two cohorts (with data collected in 1991 and 2006) [129]. These authors also found that

between these two groups, menarche was not significantly shifted, but Tanner stage 2 breast development occurred at a significantly younger age in the 2006 cohort compared to the 1991 cohort. These epidemiological studies suggest that the timing of thelarche may be advancing without a comparable change in timing of menarche. Our study shows that vaginal opening may be advancing in response to a postnatal estrogen challenge, whereas the female mammary gland development shows no changes in developmental timing, opposite of the human trends. This suggests that further studies are needed to evaluate other aspects of HPO axis function, also probing the windows of exposure and the doses administered. It is possible to speculate that changes to the doses and timing of exposure may induce significant effects at the level of the mammary gland; alternatively, chemicals with different modes of action (e.g., not estrogen receptor agonists) could be responsible for the human trends.

Several prior studies evaluating the effects of developmental xenoestrogen exposures on postnatal estrogen challenges have focused on the disruption of the endocrine axis, using ovariectomizing females. One study revealed that perinatal exposures to BPA heightened the sensitivity of the mammary gland to an estrogen challenge [95]. In our study, our female mice were intact because we were interested in the interaction of prenatal and postnatal estrogen exposures in a setting more relevant to humans; humans are exposed throughout life to a plethora of hormonally-active chemicals including estrogenic pharmaceuticals, and then later in life experience exposure to endogenous hormones. Most girls and women retain their ovaries throughout life. Thus, the purpose of our study is not to evaluate how an otherwise inactive HPG axis is affected, but it is to understand the

interactions of prenatal and postnatal estrogen exposures, where individuals are otherwise physiologically normal.

Our recent study exposed pregnant mice to BPS from pregnancy day 9 through the entire lactational period (LD21) [125]; assuming transfer of BPS in both maternal blood (in utero) and milk (postnatally), the offspring were exposed from embryonic day 9 until weaning. The current study utilized a shorter period of exposure, focused on the pregnancy/gestational period. This shorter period is still expected to cover the critical developmental period for the mammary gland [130]. In both studies, we observed no significant effects of BPS exposure on female mammary gland at puberty. Yet, the male mammary gland is affected by BPS exposure, specifically in the right mammary gland, and the female demonstrated abnormalities that manifest in adulthood. This may suggest that the relatively high levels of circulating hormones in the female at puberty are sufficient to 'mask' the effects of prenatal xenoestrogen exposures. It may also mean that mammary gland morphology is not the correct endpoint to reveal the effects of BPS at puberty; ongoing studies evaluating gene expression will help to elucidate if molecular endpoints are more sensitive.

Completing this study revealed a number of important future directions. Additional studies are needed to assess the long term implications of this "two hit" exposure paradigm to evaluate which adverse effects will manifest later in life in adulthood; specifically females challenged with EE2 during puberty should be raised to adulthood, and other females should specifically be challenged in adulthood, similar to the hormone exposures experienced when women use pharmaceutical birth control. Additional, perhaps narrower, critical windows of development could also be assessed, so we can narrow our

understanding of the crucial duration where exposure to environmental chemicals could have the most negative adverse outcomes. Finally, to compare our results with studies in BPA, an interesting future study could also examine how BPS-exposed females respond to an EE2 challenge if they are ovariectomized. This could lead us to better understand the effects of perinatal xenoestrogen exposures on function of the hypothalamus-pituitary-ovarian axis and determine which part of the axis is targeted by BPS. Similarly, we could orchiectomize the males to evaluate how the absence of androgens influences the response of the mammary gland (and other hormone-sensitive organs) to a postnatal estrogen challenge.

CHAPTER 3

DEVELOPMENTAL BPS EXPOSURE AND RESPONSE TO ESTROGEN IN ADULTHOOD

Introduction

In the first series of experiments completed for this thesis, the two-hit model was utilized to evaluate the effects of developmental BPS exposures on both male and female mice at puberty. Specifically, mice were exposed to BPS from gestational day 9 through postnatal day (PND) 2 (the perinatal period) and then provided a postnatal estrogen challenge from PND21 until PND31, overlapping the onset of puberty. Relatively few effects of either the perinatal or the postnatal treatments were observed on the females, including growth and developmental parameters of the mammary gland. In completing that study, we acknowledged that additional studies were needed to assess the long-term implications of this "two hit" exposure paradigm to evaluate whether adverse effects of developmental BPS exposure would manifest later in life. We were specifically interested in challenging female mice in adulthood with estrogen; this would simulate the hormone exposures experienced when women use pharmaceutical birth control.

In our recent study, we evaluated mammary gland development in female mice exposed to 2 or 200 µg BPS/kg/day during gestation and the perinatal period [125]. Once these females reached adulthood, they displayed increased ductal branching and alveolar buds, increased density of all mammary epithelium, retained TEB-like structures, and an increased incidence of intraductal hyperplasia. Collectively, these results suggest that early life BPS exposures induce abnormal mammary gland outcomes that are visible in

adulthood, and some of these effects (e.g., retained TEB-like structures) might indicate that these animals would have a heightened responsiveness to ovarian hormones. Because TEBs are one of the sites where carcinomas originate [131], we were especially interested in evaluating whether BPS-treated animals developed pre-cancerous lesions.

A prior study evaluated ovariectomized middle-aged female rats that were given estrogen replacement therapy for three months following exposure to 0.5 or 50 µg BPA/kg/day during development [96]. The BPA-exposed rats developed hyperplasia in the mammary gland in response to the estrogen challenge, consistent with a sensitization of the gland to early life BPA exposure that ultimately heightens the response of the mammary tissue to adult estrogen exposures.

Here, in our on-going study, we have evaluated the effects of perinatal xenoestrogen exposures on the response of the mammary gland and other hormone-sensitive endpoints after an adult estrogen challenge in early adulthood (3 months) as well as in later adulthood (9 months). We specifically focused on mammary gland and reproductive tract outcomes in female mice including the weight of estrogen-sensitive organs, incidence of gross abnormalities in organ appearance (e.g., ovarian cysts), and abnormal behavioral observations. The morphology of the adult mammary gland and its response to the adult estrogen challenge is currently under evaluation.

Methods & Materials

Treatment of Mice with Test Chemicals

Timed pregnant female CD-1 mice (Charles River Laboratories, Raleigh, NC) were individually housed (until parturition) in polysulfone cages with food and water (in glass bottles) provided ad libitum. The animals were maintained in temperature and light controlled (12h light, 12h dark, lights on at 0800 h) conditions at the University of Massachusetts, Amherst Central Animal Facility. All experimental procedures were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

Mice were allocated to treatment groups using statistical software to equalize body weight at the beginning of the dosing period. From pregnancy day 9 to lactational day 2, dams were orally dosed with BPS, EE2 or vehicle (Tocopherol Stripped Corn Oil) (Figure 3.1). The chemicals were diluted in corn oil at a concentration administration of 1 µg oil for every 1 g of body weight. The diluted solutions were designed to deliver 2, 200 or 2000 µg BPS/kg/day or 1 µg EE_2/kg/day. In total, the number of pregnant dams in each of the treatment groups was: control, n=11; 2 µg BPS/kg/day, n=10; 200 µg BPS/kg/day, n=12; 2000 µg BPS/kg/day, n=10; 1 µg EE2/kg/day, n=10. Dams were trained to drink oil from the tip of a pipette, allowing chemicals to be administered orally while avoiding the overt stress of gavage. Doses were adjusted daily for body weight. Dams delivered naturally (birth designated lactational day 0 [LD0]). Litters were not culled.

At PND21, pups were moved to new cages, separated by sex, and weaned. Starting on PND80 and on PND260, two females from each litter were selected at random for the early adulthood estrogen challenge and the later adulthood estrogen challenge, respectively. One

female was assigned to receive vehicle, and one was assigned to receive an estrogen challenge (Figure 3.1). Each adult female was orally dosed via a pipet with either vehicle (Tocopherol Stripped Corn Oil) or 1 µg EE2/kg/day for 10 days. Doses were adjusted daily for body weight.

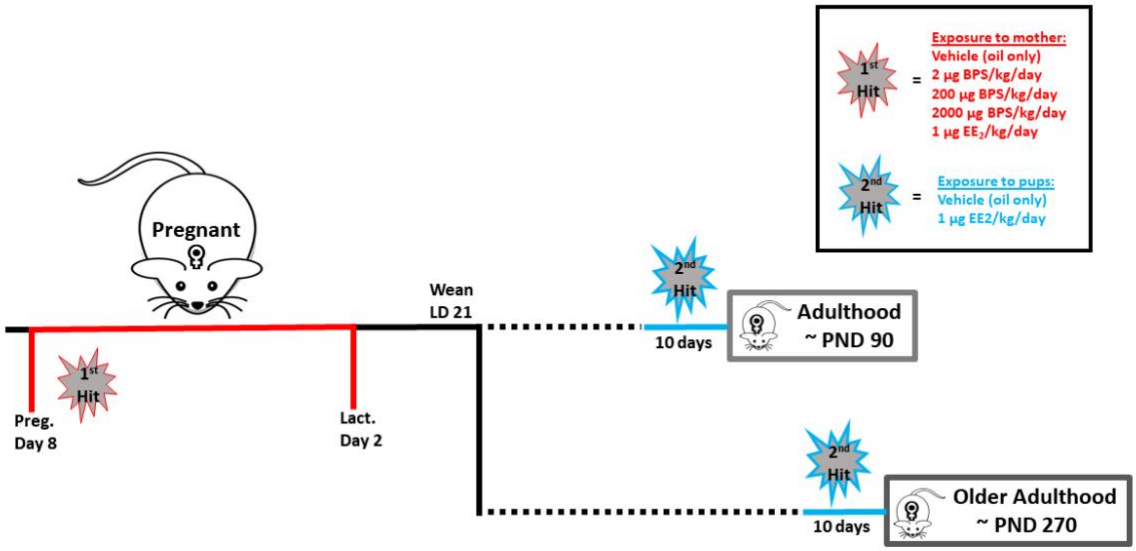

Figure 3.1. Experimental design schematic. Here, first-hit exposure to the mouse is a developmental exposure from gestational day 8 through postnatal day 2. The second-hit exposure (an estrogen challenge) is a 10 day treatment in either early adulthood (approx. PND 80-90) or older adulthood (approx. PND 260-270).

Necropsies and Dissections

After 10 days of treatment (oil or EE2), adult female offspring were euthanized by CO_2 inhalation followed by decapitation. Trunk blood was collected and serum was isolated. A vaginal smear was collected to verify estrus cycle stage, and anogenital distance was measured with calipers. The left and right fourth inguinal mammary glands were isolated using standard dissection methods. The right mammary gland was spread on a positively charged slide and fixed in neutral buffered formalin for whole mount processing. The left

mammary gland was drop-fixed in neutral buffered formalin for later paraffin embedding and histological analysis. The pectoral/thoracic mammary tissue was also collected and snap frozen for RNA. The uterus, ovaries and liver were weighed using an analytical balance. One horn of the uterus was fixed in neutral buffered formalin, while the other uterine horn was snap frozen for RNA. Tissues with gross morphological defects were collected for histological evaluations.

Carmine Alum Whole Mount Staining and Whole Mount Morphometric Analysis

After 24-48 hours of fixation at room temperature, whole mount mammary glands were washed with phosphate buffered saline and stored in 70% ethanol. For staining, whole mounts were dehydrated through a series of alcohols and defatted with toluene. Next, the glands were stained overnight with carmine, dehydrated through a series of alcohols and xylene, and then placed in k-pax heat bags with methyl salicylate, an optically beneficial preservative agent.

Whole mounts mammary glands from adult females were imaged using a Zeiss Axio Imager dissection microscope. Using Zen Pro software, the whole mounts were quantitatively analyzed at a magnification of 35x using the region immediately posterior to the central lymph node. A 180 cross-hair grid was placed over each image, and morphological structures including ducts, terminal ends, and alveolar buds were counted at each crosshair. The volume fraction of each structure was then calculated in relation to the whole image (# of crosshairs falling on structure/ # total crosshair on mammary gland). In addition, other images of ‘abnormalities’ were taken (e.g., beaded ducts, which have been previously shown to be intraductal hyperplasias [132]).

Embedding/Sectioning and Staining of Fixed Tissue

Following fixation, mammary glands and other tissues collected with gross morphological abnormalities were washed and processed through a series of graded alcohols followed by vacuum infiltration of paraffin. After tissues were embedded in paraffin, the blocks were trimmed and stored at -20°C. Using a rotary microtome, tissues were sectioned at a thickness of 5µm, floated on a hot water bath and collected on positively charged glass slides.

Sections were processed through a series of xylenes to deparaffinize the tissues, followed by a graded alcohol series (100%, 95%, 70%) to rehydrate the tissue. Samples were stained with hematoxylin and eosin (approx. 1 min for hematoxylin, 4-5 min for eosin) and dehydrated through an alcohol series and xylene. Samples were coverslipped with permount solution. Samples were evaluated using a Zeiss Oberserver.Z1 inverted microscope and 20x and 40x objectives. Images were collected with a Zeiss high resolution color camera.

Vaginal Smear Staining

At time of necropsy, a vaginal smear was collected on a glass slide. Smears were rapidly fixed in formalin and processed through a graded series of alcohols. Samples were stained with hematoxylin and eosin, dehydrated, and coverslipped with permount. Smears were viewed with a Zeiss Oberserver.Z1 microscope and scored as one of four estrus stages.

Statistical Analyses

SPSS v24 was used for all statistical analyses. All data were collected by experimenters blind to treatment group. For all normally distributed data collected from pups, two-way ANOVA (with prenatal and postnatal treatments representing independent variables) followed by Fisher's LSD posthoc were used to assess the differences in morphological features between treatment groups. Categorical data were evaluated with Chi Square tests. Results were considered significant at $p < 0.05$. All data presented in graphs represent mean ± SEM.

Results

Neither prenatal xenoestrogen exposure or a postnatal estrogen challenge affects AGD/AGI in female in early or late adulthood

Prior studies have shown that AGD in male but not female rodents is affected by developmental exposures to estrogenic or anti-androgenic chemicals. Although AGD continued to get longer in the females with age (as expected because of their larger body size), neither AGD nor AGI were altered in adult females based on prenatal exposure to xenoestrogens or the postnatal EE2 challenge (Figure 3.2)

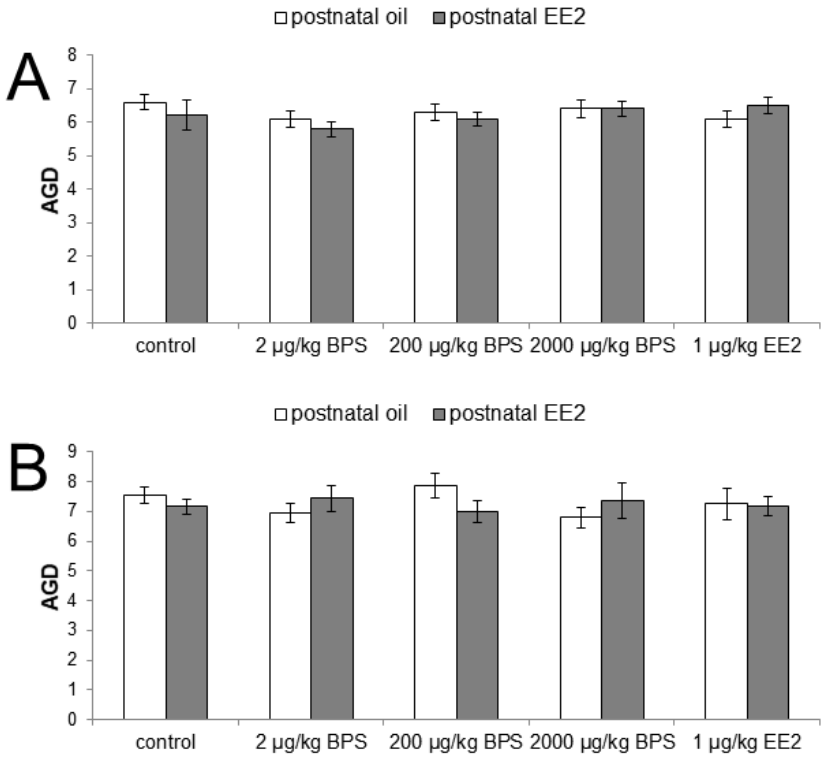

Figure 3.2. Anogenital distance (AGD) was unaffected by developmental xenoestrogen exposure or a postnatal estrogen challenge. A) AGD in females at PND90. B) AGD in females at PND270. Anogenital index, which accounts for differences in body weight, was also not affected by treatment (data not shown).

Low dose developmental BPS exposure induced modest effects on body weight in early and late adulthood

Female offspring were weighed daily from PND 80-90 (young adulthood) or PND 260-270 (older adulthood) during the administration of the postnatal estrogen challenge (or the vehicle control). At PND90, there were no differences in body weight based on developmental xenoestrogen exposure or the postnatal EE2 challenge (Figure 3.3A). In later adulthood, there were modest decreases in body weight observed in the females perinatally exposed to 2 or 2000 μg BPS/kg/day (Figure 3.3B). Although these differences were not statistically significant, they are similar to the effects of BPS on body weight that we observed previously in BPS-exposed pups [49]. The postnatal estrogen challenge did not affect body weight.

The postnatal estrogen challenge increased uterine weight in young adult female controls, but not xenoestrogen exposed females

Estrogen exposure is known to increase the weight of the uterus in ovariectomized female rodents [133]; this uterotrophic response is a predictable effect of estrogens when the hypothalamic-pituitary-ovarian axis is not intact (e.g., due to ovariectomy), but is often not evaluated when the ovaries are present. In the female mice at PND90, 2-way ANOVA revealed a significant effect of postnatal treatment, but not perinatal treatment, on uterine weight. Posthoc statistics noted an increased uterine weight in controls after a postnatal estrogen challenge, but no significant effect in any of the BPS-treated groups (Figure 3.4A). The effect of postnatal EE treatment on uterine weight was not observed in later adulthood (Figure 3.4B).

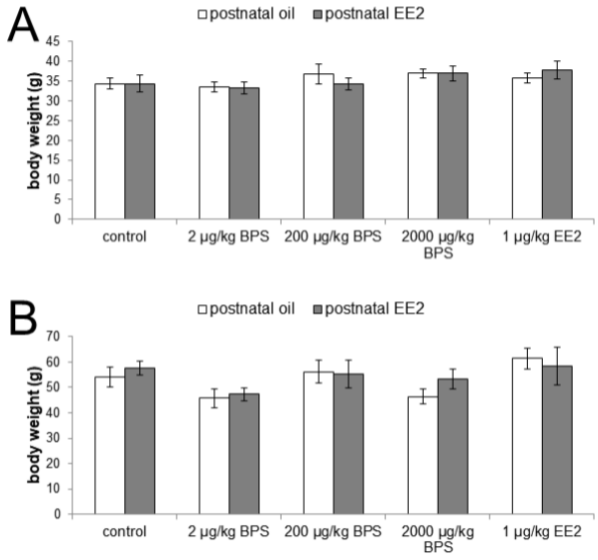

Figure 3.3. Body weight in female adults at PND 90 (A) or PND 270 (B). Although there were modest decreases in body weight in females perinatally exposed to 2 or 2000 µg BPS/kg/day in late adulthood, these were not statistically significant.

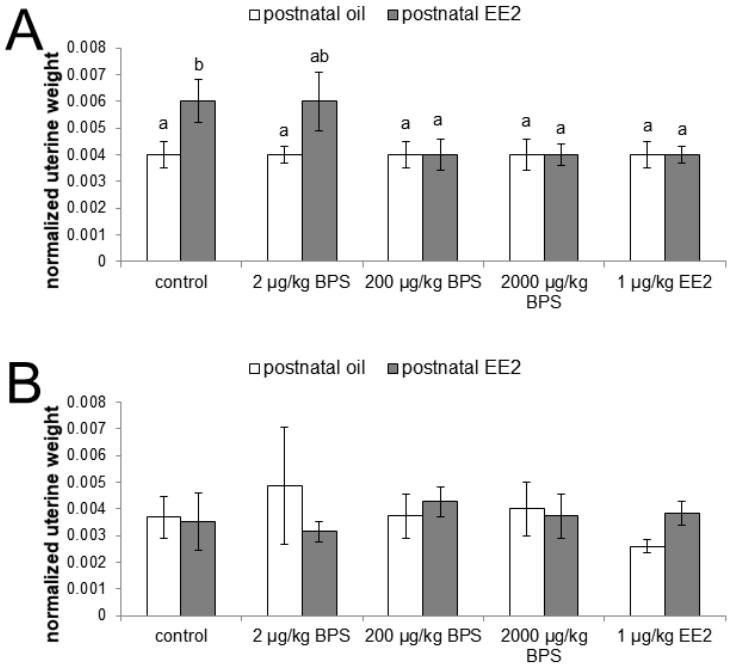

Figure 3.4. Normalized uterine weight was increased in early adult female controls, but not BPS-treated females, by a postnatal estrogen challenge. A) Uterine weight at PND 90. Different letters indicate significant differences between groups ($p<0.05$, Fisher's posthoc after significant 2-way ANOVA). B) Uterine weight was not affected by developmental xenoestrogen exposure or a postnatal estrogen challenge at PND270.

Ovarian weight is highly variable in early and late adulthood

Weight measures of the right and left ovary were taken separately at both early and late adulthood. These weights were found to be very consistent in the control females, whereas ovarian weights were highly variable in BPS-treated females. In early adulthood, the left ovarian weights had a large variance in the 200 and 2000 μg BPS/kg/day and the developmental EE2 treatment groups; this was typically because of a single very large ovary within each of these groups (Figure 3.5A). The right ovarian weights at PND90 were more consistent and 2-way ANOVA revealed a significant effect of postnatal treatment, but not prenatal treatment ($p<0.05$). Posthoc evaluations revealed significant decreases in ovarian weight associated with the postnatal EE2 challenge in the animals developmentally exposed to 200 μg BPS/kg/day (Figure 3.5B). Because these effects were only seen in the right ovary in early adulthood, and effects on ovarian weight were not observed in later adulthood (Figure 3.5C,D), it is difficult to evaluate the relevance of this effect.

Ovarian cysts, a sign of reproductive aging, were observed in early and late adulthood

Previous studies have reported the presence of ovarian cysts, a sign of reproductive aging, in mice developmentally exposed to BPS [134]. Ovarian cysts (both clear and bloody) were observed in mice at both PND90 and PND270 (Figure 3.5E). At PND90, ovarian cysts appeared to be induced by postnatal EE2 treatment in the controls and low BPS group, but not in the mid and high BPS groups (Table 3.1); bloody ovarian cysts were relatively rare (Table 3.2). This pattern was not observed in older adult females, where ovarian cysts were more common in all groups except the control females perinatally

exposed to EE2 (Table 3.3). Interestingly, bloody ovarian cysts were most common in females perinatally exposed to 200 µg BPS/kg/day after a postnatal EE2 challenge (Table 3.4).

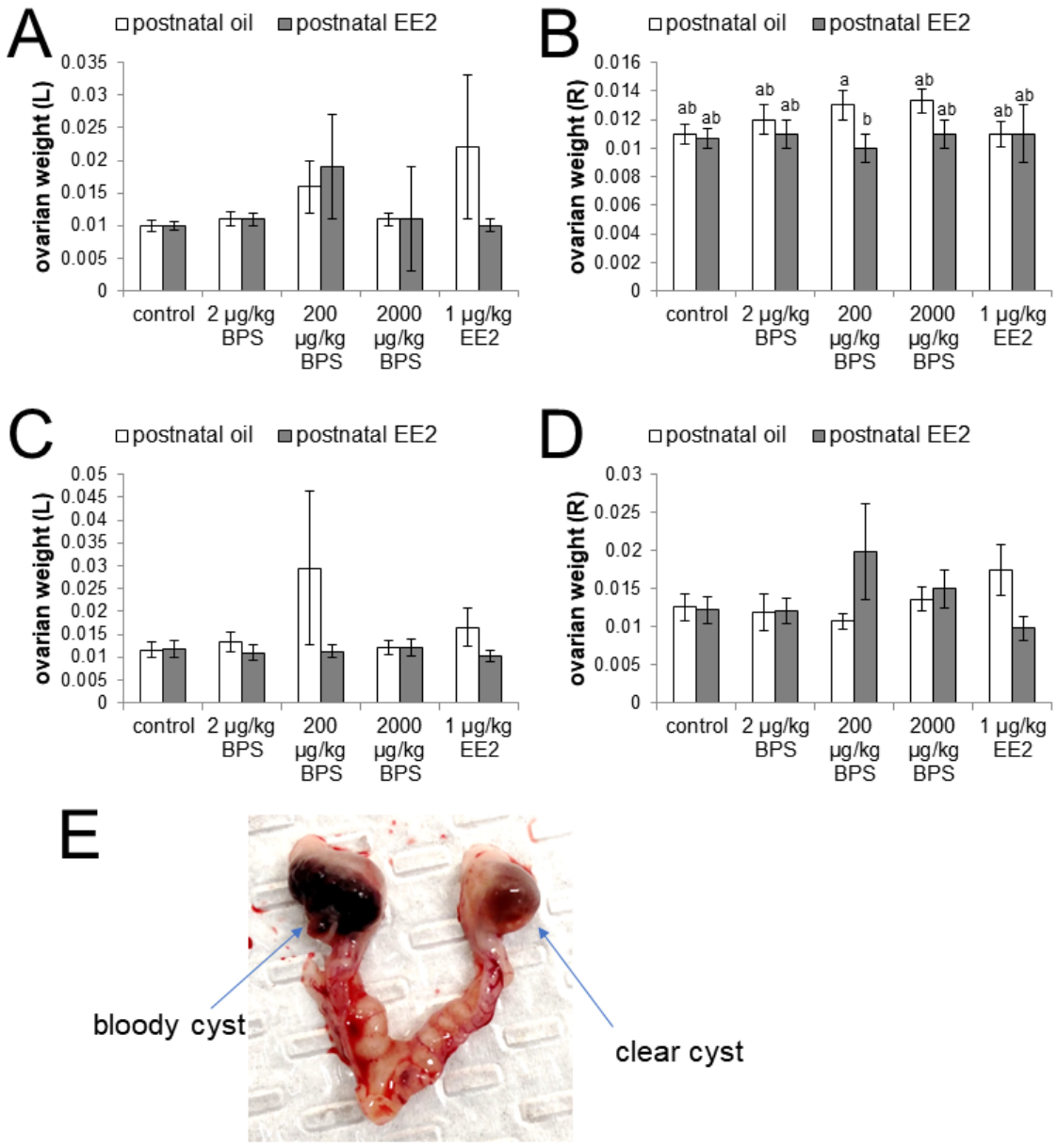

Figure 3.5. Ovarian weight in female mice in early and late adulthood. A, B) Weight of the left and right ovaries in early adulthood (PND90). C, D) Weight of the left and right ovaries in late adulthood (PND270). E) Image of bloody cyst on right ovary and clear cyst on left ovary. Different letters indicate significant differences between groups ($p<0.05$, Fisher's posthoc after significant 2-way ANOVA).

Table 3.1. Incidence of ovarian cysts at PND90

	postnatal oil	postnatal EE2
control	1/10 (10%)	4/9 (44%)
2 µg/kg BPS	1/9 (11%)	4/9 (44%)
200 µg/kg BPS	3/11 (27%)	1/11 (9%)
2000 µg/kg BPS	0/7 (0%)	0/8 (0%)
1 µg/kg EE2	0/8 (0%)	2/8 (25%)

Table 3.2. Incidence of bloody ovarian cysts at PND90

	postnatal oil	postnatal EE2
control	1/10 (10%)	1/9 (11%)
2 µg/kg BPS	1/9 (11%)	1/9 (11%)
200 µg/kg BPS	3/11 (27%)	1/11 (9%)
2000 µg/kg BPS	0/7 (0%)	0/8 (0%)
1 µg/kg EE2	0/8 (0%)	2/8 (25%)

Table 3.3. Incidence of ovarian cysts at PND270

	postnatal oil	postnatal EE2
control	3/9 (33%)	0/9 (0%)
2 µg/kg BPS	1/8 (13%)	4/8 (50%)
200 µg/kg BPS	3/10 (30%)	8/10 (80%)
2000 µg/kg BPS	3/7 (43%)	1/7 (14%)
1 µg/kg EE2	0/6 (0%)	2/6 (33%)

Table 3.4. Incidence of bloody ovarian cysts at PND270

	postnatal oil	postnatal EE2
control	2/9 (22%)	0/9 (0%)
2 µg/kg BPS	1/8 (13%)	3/8 (38%)
200 µg/kg BPS	1/10 (10%)	4/10 (80%)
2000 µg/kg BPS	0/7 (0%)	0/7 (0%)
1 µg/kg EE2	0/6 (0%)	1/6 (17%)

Neither developmental xenoestrogen exposure or postnatal EE2 exposure altered liver weight in adult females

Liver weight is often used as an index of toxicity; an enlarged liver can be a sign of a systemic health insult. There were no changes in normalized (to body weight) liver weight based on either perinatal xenoestrogen exposure or postnatal estrogen treatment at either early (Figure 3.6A) or late adulthood (Figure 3.6B).

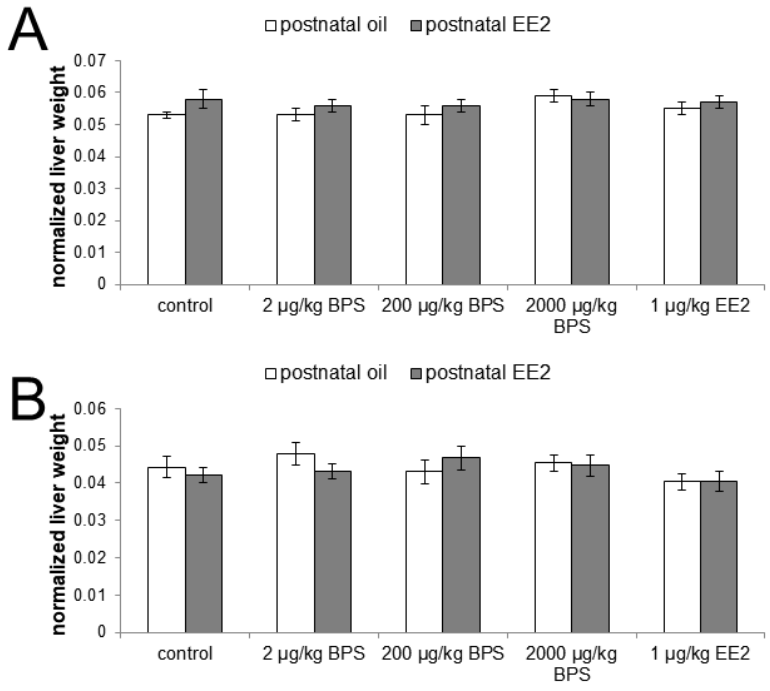

Figure 3.6. Liver weight was unaffected by perinatal or postnatal treatment. A) Normalized liver weight at PND 90. B) Normalized liver weight at PND 270.

Female mice develop gross morphological abnormalities in late adulthood (PND270)

Visceral organs were examined from female mice at necropsy. At PND90, no overt abnormalities were observed. In contrast, females in late adulthood (PND270) exhibited visible abnormalities at tissue collection for uterus, ovary, liver, mammary lymph node,

kidney, spleen as well as a case of systemic edema and two tumors (see Figure 3.7A for summary). All abnormal tissues were collected in fixative, sectioned, and stained with H&E for pathology analysis (see Figure 3.7B for examples). Further analysis and pathology diagnosis of these tissues is currently on-going.

A

	control + oil	2 µg/kg BPS + oil	200 µg/kg BPS + oil	2000 µg/kg BPS + oil	1 µg/kg EE2 + oil	control + EE	2 µg/kg BPS + EE	200 µg/kg BPS + EE	2000 µg/kg BPS + EE	1 µg/kg EE2 + EE
abnormal gross morphology of the uterus		1			1	1	1	1	1	
abnormal gross morphology of ovary		1	1	1						
edema								1		
enlarged spleen					1			1		
liver tumor					1					
enlarged mammary lymph nodes	2									
abnormal gross morphology of kidney		1			1					
other tumors			1		1					

B

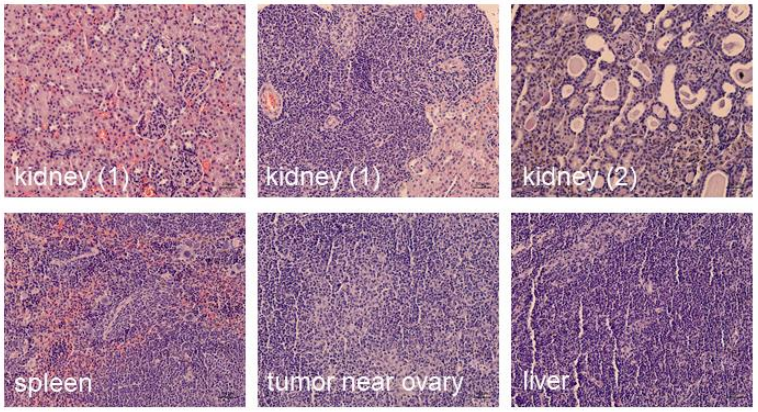

Figure 3.7. Gross abnormalities observed in females in late adulthood. A) Chart quantifying specific morphological abnormalities in each treatment group. This table is split by treatment group, where animals postnatally exposed to vehicle/oil are shown in green, and animals postnatally exposed to an estrogen challenge are shown in orange. B) Histological images of select abnormal tissues collected from female mice at PND270. As indicated by labels on the panels, abnormal tissue morphology was observed in the kidney, spleen, a tumor near the ovary, and the liver. Additional tissues continue to be evaluated.

Initial evaluations of the whole mount mammary glands are consistent with BPS-induced changes in tissue differentiation

Ongoing quantitative evaluations of mammary gland morphology are examining whether developmental xenoestrogen exposure and/or a postnatal estrogen challenge alters the morphology of the mouse mammary gland. Undergraduate assistants have helped us to quantify structures in the adult mammary gland including ducts, terminal ends, and alveolar buds. Initial evaluations indicate effects of developmental BPS exposures that are consistent with our prior study; perinatal BPS exposure appears to increase branching and alveolar buds in adult animals (Figure 3.8). However, we noted discrepancies in the quantification of the animals at PND90 and PND270, indicating that quantification of these time points should be completed by a single experimenter. This will be completed, again using researchers that are blind to treatment groups.

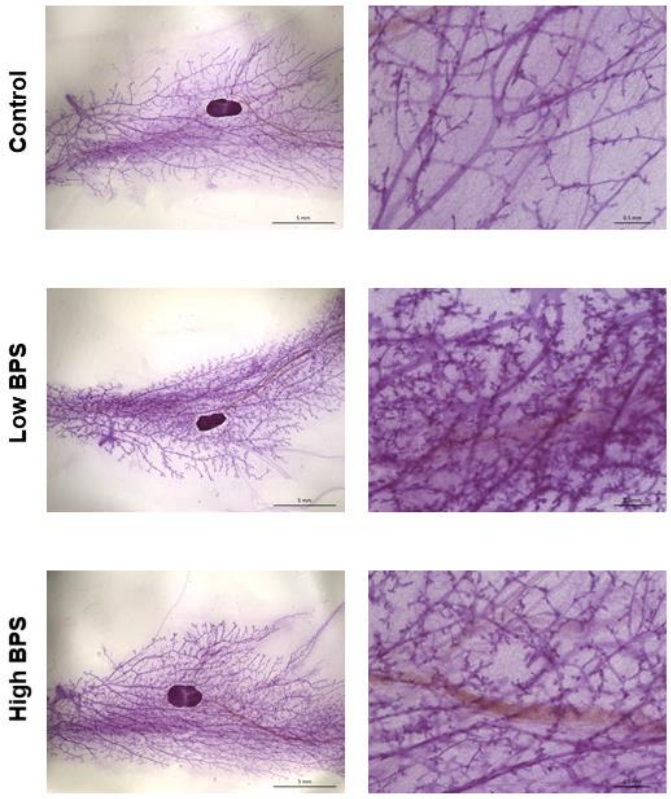

Figure 3.8. Low and high magnification images of mammary glands at PND90.

Behavioral observations prior to EE2 challenge revealed stereotypies and hyperactivity in BPS treated animals

Animal behavior observations were made between puberty and the administration of the EE2 challenge in adulthood. Stereotypies, typically characterized as continuous back-flipping, were observed only in animals developmentally exposed to BPS. Similarly, hyperactivity such as highly repetitive circling behavior, was also only observed in BPS treated animals. Other generalized anxiety behaviors, including barbering and excessive food chewing, were observed in all treatment groups (Table 3.5).

Table 3.5. Behavioral observations made in adult females prior to EE2 challenge.

	stereotypies (e.g., backflipping)	**hyperactivity (e.g., circling)**	**anxiety (e.g., food chewing, barbering)**
control	0/11	0/11	4/11
2 µg/kg BPS	2/10	1/10	5/10
200 µg/kg BPS	1/12	3/12	3/12
2000 µg/kg BPS	1/10	3/10	1/10
1 µg/kg EE2	0/10	0/10	4/10

Discussion

This study examined the effects of female CD-1 mice developmentally exposed to xenoestrogens, followed by a postnatal estrogen challenge for 10 days in early or late adulthood. Evaluating the effects of this two-hit model at adulthood was important because we wanted to evaluate how developmental BPS exposure would affect females past the age of puberty, at a time when pharmaceutical estrogen use is common [53, 135]. Our interest in adult exposures to estrogens stemmed from the rationale that many women utilize pharmaceutical estrogens in prescription birth control, and some women utilize hormone replacement therapy to treat menopausal symptoms. Both are additional sources of exogenous estrogen, and it is not known if developmental xenoestrogen exposures would disrupt the responsiveness of the female to pharmacologically relevant doses of EE2. Importantly, either under- or over-responsiveness to an adult estrogen challenge would raise concern for adults. Over-responsiveness might indicate an increased susceptibility to diseases that are associated with estrogen exposure like breast cancer [81]. Under-responsiveness might indicate a decreased protection from diseases typically associated with pregnancy.

Here, we assessed effects of developmental xenoestrogen exposure (BPS, EE2) on hormone-sensitive endpoints, as well as the response of those endpoints to a subsequent estrogen challenge. Specifically, we evaluated morphological characteristics of the adult female mammary gland, weights of reproductive organs (uterus, ovaries), liver weight, body weight, anogenital distance, and behavioral outcomes. Overall, there were modest effects of developmental BPS exposure on estrogen-sensitive outcomes. For example, there were non-significant effects of BPS on body weight in females perinatally exposed to either

the low or high dose of BPS, only in later adulthood. Furthermore, we also noticed that stereotypies and hyperactivity were observed only in the perinatal BPS groups, although the incidence of these behaviors was not statistically significant. We only evaluated behaviors leading up to administration of the EE2 challenge, so we cannot address whether these behaviors were increased or attenuated by postnatal estrogen.

Mammary gland morphometric analysis also showed that animals developmentally exposed to BPS have an increase in alveolar buds at early and late adulthood. However, this analysis is on-going and is not complete. We did note the presence of gross morphological abnormalities at PND270. These lesions have been extensively imaged in the whole mount mammary glands, but they need to be excised, embedded, and sectioned for pathological analysis. We believe this is a worthwhile approach because our prior study demonstrated that perinatal BPS exposure increases the incidence of intraductal hyperplasias [125], and unpublished data from the Fenton laboratory at NIEHS suggests that BPS can induce frank carcinomas in the mammary glands of CD-1 mice (Sue Fenton, personal communication). Evaluations of additional adult endpoints (e.g., qPCR of estrogen-sensitive genes) will also need to be completed in the future.

There was some evidence that developmental BPS exposure disrupted the female's response to a postnatal EE2 challenge. In early adulthood, uterine weight was increased in controls given an EE2 challenge, but this same induction was not observed in BPS-treated females, suggesting an attenuation of the effects of postnatal estrogen exposures. Importantly, this effect of estrogen on uterine weight was no longer observed in later adulthood, suggesting it should be evaluated in greater depth. Similarly, there was a decrease in the weight of the left ovary in BPS-treated females at early adulthood

associated with postnatal EE2 exposure (only significant at 200 μg BPS/kg/day, but a similar pattern observed in other dose groups); this same effect was not observed in the right ovary, and females in late adulthood did not show any effect on ovarian weight. These results are puzzling, especially because there is no explanation for why left ovarian weight is more variable in the BPS-treated females.

There were additional outcomes that were unaffected by either perinatal xenoestrogen exposures or the postnatal estrogen challenge. For example, AGD (and AGI) were unaffected, perhaps unsurprisingly as most studies conclude that males are more susceptible to this kind of disruption. Our results also showed no effects of treatment on liver weight in early or late adulthood. This is also not surprising, because the doses we selected are below the toxicological No-Observed-Adverse-Effect-Level (NOAEL) dose, and thus are not anticipated to induce overt toxicity.

One potentially sensitive endpoint that was interesting was the appearance of ovarian cysts in the females. In early adulthood, ovarian cysts were rare in control females (10%), but increased by the EE2 challenge (44%). A similar pattern was observed in the low BPS-treated group (from 11% to 44%) and the developmental EE2 group (from 0% to 25%). This pattern was not observed in females from the mid-BPS dose group, where EE2 treatment reduced the number of ovarian cysts (27% to 9%) or the high BPS dose group, where ovarian cysts were not observed. This pattern was also not observed in late adulthood. At PND270, without a postnatal EE2 challenge, all groups except developmental EE2-treated group displayed ovarian cysts. The incidence of cysts increased in the low and mid-BPS treated groups after a postnatal EE2 treatment, but decreased in the controls and high BPS dose group.

Our results also revealed some gross morphological changes, which raises concern about the possibility that developmental BPS exposure may induce 'rare' events. Currently, we are evaluating histology for tissues, including, liver, kidney, spleen, tumors, ovary, and uterus. An evaluation from a pathologist will be needed to assess these abnormalities. Similarly, we have started to evaluate gross morphological lesions in the whole mount mammary glands but need to dissect these to evaluate them histologically. We have noticed structures like beaded ducts, hyperproliferative alveolar buds, and other abnormal structures that need to be examined in depth.

This study was focused on evaluating phenomena, but not mechanisms. At this time, we cannot conclude how developmental BPS exposure alters the response of some endpoints to a postnatal EE2 challenge. For many of these endpoints, the effects from this two-hit model are specific to the tissue rather than generalizable to the individual. For example, developmental xenoestrogen exposure may be masking the increase of uterine weight that is observed in the controls after an EE2 challenge whereas in the ovary, the postnatal EE2 treatment reveals effects in the ovary that are not observed in controls. Thus, future studies evaluating the mechanism of how developmental xenoestrogen exposures alter the response of animals to postnatal challenges will need to examine different organs and cell types separately.

In our study, endpoints such as ovarian cysts, preliminary mammary gland observations, and behavioral outcomes are important because these endpoints may be predictive of adverse outcomes in humans. For example, altered mammary gland morphology may indicate an increased risk of cancer [81], whereas the presence of stereotypies may indicate psychological perturbations [61].

Continuation of this study will allow us to understand the effects of the "two hit" (environmental EDC + estrogen) model in female mice at adulthood. This study will also provide more insight into critical windows of development and effects that may onset later in life. Future studies can assess different doses of BPS and manipulate the current paradigm to address if developmental xenoestrogen exposure alters the HPO axis in ovariectomized female mice. This would help us understand the effects of BPS on this central axis, as well as peripheral estrogen sensitive tissue, like the mammary gland. Future studies should specifically address the effects of developmental BPS exposure in the absence of endogenous estrogen (due to ovariectomy) and how a postnatal EE2 challenge alters growth and differentiation parameters.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Developmental Xenoestrogen Exposures and the Mammary Gland

Recent work from our lab demonstrated that development of the mouse mammary gland is altered by perinatal BPS exposures [125]. Effects observed at PND24, the very beginning of puberty, suggest that BPS exposure results in larger TEBs, while simultaneously diminishing proliferation throughout the gland. The adult mammary glands were also significantly different in the BPS treated females compared to controls. Overall, there was more development of the gland, which is attributable to an increased number of terminal ends and alveolar buds (ABs). In contrast, there were retained TEBs, suggesting a delay in development. Although this seems contradictory, the various aspects of mammary development (development of ABs and regression of TEBs) are influenced by different signals within the mammary tissue as well as hormonal controls.

In our prior study, there were no significant effects of developmental BPS exposure observed at the height of puberty (PND32-35). This result is consistent with our observations in the current study for pubertal females (evaluated at PND31). Why might we observe effects of BPS at the beginning of puberty and in adulthood, but not at the height of puberty, between these time points? The absence of significant effects at the height of puberty may be a result of the doses of BPS that were administered; higher doses might induce effects that are striking enough to produce visible effects at a time of significant tissue remodeling. More likely is the possibility that the large amount of natural variability makes detecting statistically significant changes at this time in development

difficult. Puberty is time when hormonal activity is high, but variable between individuals. For this reason, it may be easiest to detect the effects of BPS in ovariectomized animals that are provided consistent supplementation of ovarian hormones, similar to what has been previously published in studies of BPA [95, 136].

Using the Two-Hit Model to Evaluate Environmental Chemicals

In this thesis, I assessed the effects of developmental BPS or EE2 exposure on the mammary gland and evaluated its response to a 'second hit' of estrogen during multiple stages of life including puberty, early adulthood, and later adulthood. Prior studies have shown that development exposures to BPA increased mammary gland sensitivity to a postnatal estrogen challenge in ovariectomized mice, and found that ovariectomized female rats developed mammary hyperplasia and benign cysts [95, 96]. For male rats, a series of studies revealed that perinatal BPA exposure, paired with an adult hormone challenge, disrupted the prostate by inducing the formation of intraepithelial neoplasia and some instances of adenocarcinoma [97, 98, 137-139].

In our study, data from pubertal male mice suggest that developmental BPS exposure alters the response of some endpoints, including the mammary gland, to an estrogenic challenge. Specifically, we found that developmental exposure to BPS had no effect on growth or TEB parameters in the right gland, but the postnatal EE2 challenge modestly increased these features in controls (typically a 2x increase), and induced striking growth (typically 4-5x increase) in males from the 200 and 2000 μg BPS/kg/day groups. In contrast, males from the 2 μg BPS/kg/day group were non-responsive to the postnatal EE2 challenge. Entirely different patterns were observed for the left mammary gland,

where a postnatal EE2 challenge induced growth in TEB, but not ductal tree parameters in controls; males from the 200 and 2000 μg BPS/kg/day groups were induced to grow in response to the EE2 challenge, whereas males exposed to 2 μg BPS/kg/day were again non-responsive for any mammary endpoint. Collectively, these results suggest complex, tissue-specific (and also left-right asymmetric) responses to both prenatal and postnatal hormone exposures in males at puberty.

In the pubertal females, we did not observe altered responses to the estrogen challenge in any BPS-treated group for any mammary endpoint, AGD, or uterine weight. This may indicate that the endogenous estrogens being produced by the females at the natural onset of puberty are sufficiently high, or variable, to mask any effects of the secondary challenge. There was a modest, but non-significant, decrease in the timing of vaginal opening in control females; this decreased timing was not observed in any BPS-treated group. In future studies, our design would include an ovariectomized group, allowing a comparison to prior BPA studies, which showed that developmental exposures to BPA sensitizes the animal to a controlled estrogen exposure at puberty.

The female adult data analysis is ongoing and appears to indicate modest changes in response to the EE2 challenge. For example, in young adults, the postnatal estrogen treatment increased the weight of the uterus in control females; this increase was not observed in the BPS-treated groups.

Traditional Risk Assessment of Environmental Chemicals Including EDCs

Risk assessment involves hazard characterization, exposure assessment, dose response assessment, and risk characterization [140]. Each of these steps has posed challenges to the

identification and evaluation of EDCs. Hazard characterization has been particularly difficult at least in part because there is no consensus definition of what constitutes an EDC among global organizations. There are also fundamental disagreements about which endpoints in studies of EDCs should be considered 'adverse' [7, 8]. EDCs are also difficult to characterize because they belong to many different chemical groups and there are still thousands of chemicals in use that have yet to be tested for endocrine disrupting properties [4]. Exposure assessment for many EDCs poses a problem because there are various exposure routes, changes in duration of exposure, unexpected exposures via consumer products, as well as an importance of timing of exposure in an individual, which is often poorly evaluated [141]. Dose-response assessment is complex for EDCs because they act through multiple modes of actions and pathways, while affecting a large number of biological processes [142]. Thus, dose-response assessment may have to be specific to the endpoint of interest for each EDC, which does not allow much room for generalization in risk assessment. Risk characterization requires a level of certainty in the three assessments prior to its own to be able to communicate the risk as well as the uncertainties due to data gaps.

During the process of hazard identification, decision-making on the safety of chemicals is dependent on the availability of regulatory toxicity studies that have been validated and standardized using guidelines developed by the Organization for Economic Cooperation and Development (OECD) or similar organizations [143, 144]. OECD guideline studies therefore provide processes for assessing the risk of chemicals that were developed to ensure comparability, reproducibility, and reliability in studies performed across laboratories. Yet, academic scientists have raised numerous concerns about the use of

OECD guideline studies to evaluate hazards. One major concern has been the reliance on endpoints like organ weight, which are unrelated to the diseases of interest in human populations that have been associated with EDC exposures [3, 7, 8, 145]. Researchers have noted that more sensitive 'upstream' endpoints, including the use of tools to evaluate molecular changes (like changes in gene expression), are more likely to reveal the effects of EDC exposures.

The mammary gland is an important endpoint to consider when evaluating chemical safety, although, it is not one that is widely used in current guideline studies. Mammary gland evaluations in OECD compliant studies usually examine a single cross-section of the gland, cut perpendicular to the skin. This means that chemical safety evaluations that have examined the effects of environmental chemicals on the mammary gland draw conclusions from a dozen or so ducts, rather than a full view of the ductal tree [79, 80, 146]. Researchers have pointed out that morphological endpoints, like the ones we have measured in whole mounts, are ignored or discounted; animals are often not kept long enough to observe mammary diseases like cancer; and mammary function (lactation) is poorly evaluated, if at all [147].

Other challenges to risk assessments have focused on dose responses and their use in risk characterization. For example, Myers et al. criticized the typical methods of testing chemicals at high doses and then extrapolating to lower doses, assuming a linear relationship between dose and response [148]. This approach fails to recognize non-monotonic responses, even though low dose exposures and non-monotonic dose responses are common for EDCs [142, 149].

Finally, risk assessments are only successful when exposures are adequately evaluated. Exposure assessments for BPA indicate that knowledge about use of EDCs in consumer products, routes of exposure, and concentrations measured in human tissues and fluids changed significantly over a decade of study. BPA was originally studied in food-contact plastics, and because it was not detected in human samples, exposures were dismissed [141]. Only when analytical methods were improved did it become clear that exposures were not only occurring, but were ubiquitous in the US population [24]. Further studies revealed the extent of BPA use in consumer products, and new sources of exposure continue to be examined. It is likely that the same trends will be observed for BPA analogues including BPS.

Regrettable Replacements

The term "regrettable replacement" refers to the concept that when one chemical is found to have deleterious effects, it is often replaced with another chemical that is later determined to be similarly harmful. The idea of substitution originates from the Hierarchy of Control in the National Institute for Occupational Safety and Health (NIOSH) recommendations for reducing injuries, illnesses, and health hazards in the workplace. [150]. "Substitution" is the second tier in the hierarchy, with the first being "Elimination" of the hazard itself. The intended use of this hierarchy pyramid was to have a plan of action in emergency situations in order to protect workers and create safe practices. Unfortunately, this approach of a simple substitution for a safer environment is flawed in the case of environmental chemicals because the replacements are poorly tested, or not tested at all prior to implementation, often resulting in unintended consequences.

In the field of EDCs, eliminating a chemical is rarely the first step taken to avoid an identified hazard. Rather, industries often replace a concerning chemical with its 'cousin', a related chemical with a similar chemical structure [34]. This allows the replacement chemical to have a similar function to the original, based on the principle that 'structure dictates function'. Many of the chemicals in our consumer products play a key role in the functionality of the product and cannot merely be removed without being replaced; this need drives the development and use of replacements that are often later revealed to be regrettable.

In addition to this obstacle, there are political discrepancies between multiple sectors such as industry, academia, and government that complicate and supersede the safety of the environment and public health. In the context that most chemicals are used, current US chemical policies imitate the traditional legal system's motto of "innocent until proven guilty" [151]. This approach towards chemical regulations has been detrimental to health and the environment and will continue to be as the production of chemicals rapidly increases. In my view, the testing of chemicals must be reformed to mitigate the popular path of substitution taken by industry to protect individuals and nature from harm.

There are a few prominent examples of these regrettable replacements throughout history. One example of a series of regrettable replacements used as pesticides is lead arsenate, which was replaced with the well-known insecticide DDT, and then later replaced with organophosphate pesticides. Lead arsenate was used in the 1800s as an insecticide and pesticide on apple orchards and other crops [152]. This chemical was found to have devastating effects on the human body, causing cancers, skin lesions, and neurological impairments. DDT received world-wide attention after Rachel Carson's *Silent Spring*,

which discussed DDT's harmful effects on wildlife including altered reproductive capability. Now, organophosphates have replaced DDT, and have been shown to be neurotoxic and harmful to wildlife by blocking the enzyme acetylcholinesterase, which negatively impacts the nervous and respiratory systems [153].

Another example is polybrominated biphenyls (PBBs) and polychlorinated biphenyls (PCBs), chemicals that were manufactured prior to the 1970s for use as flame retardants in furniture and electronics, among other uses [154]. PBBs and PCBs have been shown to persist in the environment; they bioaccumulate in humans and wildlife. People are mostly exposed to PBBs and PCBs through food. These chemicals have effects on the liver, thyroid, neurodevelopment, and they are possibly carcinogenic. In replacement of these PBBs and PCBs, polybrominated diphenyl ethers (PBDEs) were introduced, which vary slightly in structure and have been shown to have similar health effects [155].

Diacetyl is a chemical that was used in popcorn butter flavoring that posed a threat to workers' lungs [156]; experimental studies in rodents found that airways were damaged due to the inhalation of this chemical [157]. In order to control for this damage to workers in the flavoring industry, diacetyl was replaced by 2, 3-pentanedione. Structurally similar to diacetyl, 2,3-pentanedione was also found to cause detrimental effects to the lung airway epithelium [158].

The most recent and widely acknowledged example is the common replacement of BPA with a range of analogues including BPS. BPA is a thoroughly studied EDC that is known for its effects on obesity, neurocognition, and reproductive endpoints [32]. BPA's presence in baby products enraged the public and a voluntary removal of BPA from some of these products was implemented [159]. This led to many consumer products being

labeled “BPA-free”, instilling a false idea that a safe alternative was being used [160]. Leonardo Trasande, a physician scientist, discussed how replacing BPA with other bisphenols is not the solution, because they too are a rising concern [161]. He suggested a natural alternative, oleoresin, that would be better for canned foods. Although it is financially less feasible, it would be less harmful than other bisphenol substitutes. Others have suggested the use of high-throughput screening assays and the addition of more sensitive endpoints to regulatory toxicity tests before allowing the replacement of harmful chemicals with other poorly studied chemicals [34, 160]. The National Research Council (NRC) and Environmental Protection Agency (EPA) are in support of “informed substitution” in order to “minimize the likelihood of unintended consequences” and suggest the use of green chemistry to better the physiochemical design of future chemicals [159].

As discussed above, conventional regulatory approaches are outdated and are not reliable for evaluating negative adverse effects; they often fail to regulate EDCs that may threaten the environment and human health [148]. Over recent years, there have been new approaches to the challenges that come with assessing toxicity and disease vulnerability of EDCs which have been well discussed and documented. These new approaches include the introduction of green chemistry principles to the development of replacement chemicals [72]. The EPA defines green chemistry as “the design of chemical products and processes that reduce or eliminate the generation of hazardous substances”. Green chemists are working to design chemicals to be more sustainable. Although traditionally chemists have not been trained to think about the biological or toxicological implications that human health and the environment may face, the introduction of green chemistry principles is

changing this. Ultimately, these approaches should help prevent the next regrettable replacement, and instead identify suitable, safer chemicals to use in place of BPA and BPS.

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