THE MOUSE MAMMARY GLAND: A TOOL TO EVALUATE THE ENVIRONMENTAL CHEMICAL BUTYL BENZYL PHTHALATE, AND APPROACHES TO IMPROVE REGULATORY TESTING

Jessica Daum
University of Massachusetts Amherst

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THE MOUSE MAMMARY GLAND: A TOOL TO EVALUATE THE ENVIRONMENTAL CHEMICAL BUTYL BENZYL PHTHALATE, AND APPROACHES TO IMPROVE REGULATORY TESTING

A Thesis Presented

By

JESSICA M. DAUM

Submitted to the Graduate School of the University of Massachusetts in partial fulfillment of the requirements for the degree of

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Public Health
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ENVIRONMENTAL CHEMICAL BUTYL BENZYL PHTHALATE, AND
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JESSICA M. Daum

Approved as to style and content by:

---------------------------------------------
Laura N. Vandenberg, Chair

---------------------------------------------
Raphael Arku, Member

---------------------------------------------
Alicia R. Timme-Laragy, Member

---------------------------------------------
Alicia R. Timme-Laragy, Graduate Program Director

---------------------------------------------
Angela De Oliveria, Department Chair, Environmental Health Science
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A special thank you to all those whose support and friendship helped me to stay focused on this project and who have provided me with the encouragement to continue despite any roadblocks in my path.
ABSTRACT
THE MOUSE MAMMARY GLAND: A TOOL TO EVALUATE THE ENVIRONMENTAL CHEMICAL BUTYL BENZYL PHTHALATE, AND APPROACHES TO IMPROVE REGULATORY TESTING

SEPTEMBER 2022

JESSICA M. DAUM, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST
M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Dr. Laura N. Vandenberg

The mouse mammary gland is an

In the first part of this thesis, we utilize the mouse model to evaluate the environmental chemical Butyl Benzyl Phthalate. Due to lack of research on female exposure to BBP, this thesis focuses on quantifying the effects of gestational exposure on the female mammary gland Here male and female parental mice were exposed before mating and through pregnancy and lactation to one of three doses of BBP or the control via oral ingestion. After weaning, offspring were sacrificed at puberty or early adulthood and evaluated for altered mammary gland morphology or hormonal receptor expression. Results indicate a persistent statistically significant increase in weight among the highest BBP dose group. Additionally, the high-dose adult treatment group demonstrated a statistically significant decrease in terminal ends. Finally, the mid-dose adult group demonstrated significantly higher expression of the progesterone receptor compared to the low and high-dose BBP groups. There were no significant findings in pubertal female outcomes.
In the second part of this thesis, we evaluate the existing OECD Extended One-Generation Reproductive Toxicity Guidelines (TG 443). First by summarizing the endpoints and outcomes evaluated in studies that implement these guidelines, and additionally discussing the current OECD recommendations for mammary gland evaluation. We conclude with outlining the remaining questions to be evaluated and further research necessary to establish that the mammary gland should be added to TG 443.
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CHAPTER 1

THE MALE MOUSE MAMMARY GLAND AND ENDOCRINE DISRUPTORS

1.1 Introduction

1.1.1 Mammary Gland

The mammary gland has important biological relevance for the growth and sustainment of mammalian species, making it an important, but relatively understudied organ (Rudel et al., 2011). The mammary gland began to gain more interest in the field of environmental health sciences after a meta-analysis of epidemiological evidence indicated that the age of onset of breast development during puberty has been rapidly decreasing since 1977 (Eckert-Lind et al., 2020). Researchers noted that this change comes alongside previous observations of the early onset of other pubertal markers, and environmental factors have been implicated in some of these trends as well (Nudelman et al., 2009, Harley et al., 2019, Sorensen et al., 2012). The main biological role of the mammary gland is lactation to sustain newborns (Marasco 2014). There is evidence that maternal exposures can also expose the fetus or newborn to environmental chemicals via the breastmilk (Fenton 2009). Thus, alterations to mammary gland development that affect its function can have adverse impacts on lactating mothers and their offspring.

The development of the mammary gland is influenced by numerous physiological processes including endocrine signaling pathways, transcription factors, and protein synthesis (Tyl, et al. 2004). The mammary gland goes through developmental changes during embryogenesis, puberty, and pregnancy/lactation (Vandenberg, 2021). In addition to these periods of physiological change, there is a final stage of the mammary gland that occurs during the menopausal transition. These later-in-life changes to the mammary
gland have become of greater interest as they occur in response to decreased estrogen production and estrogen signaling from the ovaries, yet the majority of breast cancer cases are detected during this time. The most likely reason for this occurrence is that cancer develops over many years, and as women approach menopause, they have been exposed to numerous xenoestrogens, carcinogens, and other breast cancer risk factors (drinking alcohol, obesity, hormonal birth control, not breast feeding, etc.) (Chlebowski, 2021, Willet et al., 2014). While cancers may have begun developing earlier in life, they may not be detectable until the menopausal transition.

In the mouse, the first stage of mammary gland develops from surface ectodermal cells which differentiate into the preliminary mammary gland tissue instead of differentiating into epidermis (Veltmaat, 2017). While some organs have very specific windows of gestational development, in the mouse, the budding mammary epithelial placode can be observed as early as embryonic day 10 and continues developing through late gestation (Veltmaat, 2017). The epithelial tissue develops, elongates and begins branching within the ventral mesenchyme throughout this period. This preliminary process is quite similar to the development of the mammary gland in humans. In humans, the first indication of mammary gland precursors are visible between 4-6 weeks of gestation as a mammary line, extending from the gonadal region up towards the neck of the embryo. While most of the mammary cells along this line will atrophy, distinct paired areas of the epidermis in the thoracic region will proliferate, contributing to solid epithelial masses. At the end of the first trimester and continuing through gestation the epithelial mass (mammary bud) invaginates into the underlying mesenchymal tissue (this occurs at approximately embryonic day 13-14 in the mouse). Epithelial tissue continues
to form the rudimentary mammary structure while surrounding mesenchymal cells differentiate into other structures of the mammary gland including fibroblasts, smooth muscle, endothelium, and adipocytes (Javed & Lteif, 2013).

During postnatal development, the mammary gland continues to grow slowly and fill the fat pad until puberty. At puberty, due to the increased rate of hormone production, the mammary gland rapidly develops making this the second sensitive window in the mammalian life cycle. Growth of the mouse pubertal gland is dependent on epithelial structures known as terminal end buds (TEBs). TEBs are comprised of highly proliferative cap cells that allow the ducts to advance through the fat pad. Branching occurs via apoptosis of epithelial cells located within the center of the ducts, and reinforcement of outer epithelial cells, creating a hollow system of branches. The final stage of development and a period of susceptibility to environmental insults is during pregnancy. Again, increased hormones signal mammary gland cells to proliferate then differentiate into alveolar structures which serve the purpose of creating milk to sustain newborns. Development of the mammary gland is tightly regulated by hormones such as estrogens, androgens, and progesterone (Vrtačnik et al. 2014). However, despite often being labeled as “sex hormones” these same hormones have major and minor roles in numerous other bodily functions of the male and female. For example, estrogen is a key player in development and health of the heart and blood vessels, bone, and the brain (Cui, Shen, and Li, 2013). Similarly, androgens have been associated with bone and muscle development as well as metabolism (Tchernof et al., 2018, Vrtačnik et al. 2014).
1.1.2 Diseases of the Mammary Gland

Specific attention is drawn to the increasing rates of breast cancer in today’s society. Breast cancer is the second most common cancer diagnosis among women, and the second leading cause of cancer death among women in the United States (DeSantis et. al, 2019). While breast cancer diagnosis has been increasing universally, women of color experience increased rates of breast cancer diagnosis specifically with poor prognosis. Research indicates there may be several underlying factors driving this disparity including differential access to medical care and screening, increased exposure to known risk factors, educational disparities, underlying comorbidities, and socioeconomic status (Curtis et al, 2007). The complexities of breast cancer disparity and diagnoses are further complicated by the various breast cancer phenotypes as well as numerous factors that have been identified to contribute to breast cancer risk. Risk factors for breast cancer include early menarche, increased serum estrogen concentrations, family history, presence of specific genetic mutations (e.g., BRCA1), consumption of a high fat diet, alcohol use, and tobacco smoking. There is also epidemiological evidence of protective factors against breast cancer such as regular exercise, and secondary prevention such as regular screening (Key et al., 2001).

Despite increasing rates of breast cancer diagnosis, death rates are declining due to medical advancements and early detection (DeSantis et. al, 2019). However, cancer can greatly impact one’s quality of life even if it is not lethal. Due to its complex development and treatment, increased breast cancer diagnoses have great implications for both global healthcare costs as well as individual cost and personal burden. In a recent study of cost burdens of cancers in the United States, researchers concluded that female
breast cancer is associated with the highest cost to the healthcare system as well as the individual (Yabrov et al, 2016). Costs to the healthcare system involve research costs, medical facility and equipment upkeep, wages, and additional supplementary expenditures. Costs to the individual can range from missed work, cost of treatment, cost of surgery, additional medication for symptoms caused by treatment. Burdens to loved ones and mental health are difficult to quantify but are also considered to be costs associated with cancer diagnosis (Yabrov et al, 2016).

It is increasingly evident that there are underlying contributors to cancer incidence aside from the identified genetic mutations and markers (Collins & Politopoulos 2011). Additionally, increased rates of diagnosis have impacts on both the global healthcare economy as well as the individual. Thus, evaluation of environmental exposures on the mammary gland is essential to understand the impact environmental factors, including chemical pollutants, can have on susceptibility or development of cancer.

1.1.3 Endocrine Disrupting Chemicals

When considering environmental exposures and the mammary gland, it is important to first understand the role of endocrine disrupting chemicals (EDCs), their environmental presence and their implications for human health. EDCs are exogenous chemicals, or mixtures of chemicals, which interfere with any aspect of hormone action (Zoeller et. al, 2012). EDCs can act as direct or indirect agonists and/or antagonists of hormone receptors. Additionally, some EDCs will bind to allosteric sites thus disrupting hormone action (Diamanti-Kandarakis et al., 2009). Furthermore, some EDCs directly affect organs responsible for the production or regulation of hormones such as the pituitary, thyroid, or ovaries.
EDCs can alter bodily function at all stages of life, and some have even been shown to have transgenerational effects (Janesick et al. 2014). Furthermore, exposure to EDCs during sensitive windows of development is associated with later in life disease outcomes such as diabetes (Velmurugan et al., 2017) or uterine fibroids (Bariani et al., 2021). Some of the most well researched implications of EDCs are their effects on male and female reproductive and developmental health (Laws et al, 2021). Alterations to male endocrine function has been associated with reduced semen quality and infertility, as well as testicular and prostate cancers. Developmental exposure to some EDCs has also shown to produce urogenital tract abnormalities including cryptorchidism (undescended testes) and hypospadias (malformations of the penis). Furthermore, some laboratory evidence has indicated signs of feminization via reduced anogenital distance, malformations of internal reproductive organs and retained nipples in male rodents exposed to EDCs. In females, EDC exposure has been associated with increased risk of cancers of the reproductive organs, decreased fertility, increased rate of ectopic pregnancy, early menarche, and early menopause. Additionally, laboratory researchers have found developmental exposures to EDCs can cause morphological changes to the female reproductive tract including changes to the ovarian follicles. While not yet fully understood, improper ovarian follicle development and function has been hypothesized to influence local hormonal balance. It is additionally associated with later in life diseases such as polycystic ovary syndrome, premature ovarian failure, uterine fibroids and altered fecundity.

EDCs have implications far greater than reduced reproductive function. EDCs have been further associated with obesity and other metabolic disorders (Heindel et al., 2017). The current theory is that these outcomes are influenced by altered developmental
programming. Obesogens, or environmental chemicals that promote obesity, work by indirectly altering the mechanistic pathways that regulate metabolic rate, glucose homeostasis, appetite, and satiety (Heindel et al. 2015). Thyroid hormone alteration and disruption of the PPARy pathways are two proposed manners of obesogenic properties of EDCs. While evidence of obesogenic properties of EDCs is growing, there is still much research necessary to understand the exact mechanisms in which these compounds act (Janesick & Blumberg, 2012).

With increasing research on environmental contaminants as well as the endocrine disrupting potential of components in the environment, for many years, there was a major gap in research when it came to defining the key characteristics of EDCs. A recent consensus statement published in Nature Reviews Endocrinology aimed to close this gap by identifying 10 key characteristics of EDCs for the basis of hazard identification (La Merrill et al., 2020). A chemical is considered to have endocrine disrupting properties if it is a receptor ligand or agonist, a receptor antagonist, or if it affects receptor expression, signal transduction, epigenetic alterations, hormone synthesis, hormone transport, hormone distribution or circulating hormone levels, alters hormone breakdown or clearance, or changes the fate of cells in hormone-responsive organs. There is increasing evidence that EDCs have effects after low-dose exposures. Low-dose effects can be defined in many ways, but this term generally refers to effects observed at exposure levels that are lower than those used in traditional toxicology studies used by regulatory agencies, including the doses evaluated in test guidelines (Vandenberg et al., 2012). Guideline studies are discussed later in this introductory chapter and in Chapter 3. If research on EDCs is only conducted using test guidelines, researchers may miss
important effects caused by low-dose exposure (Vandenberg et al., 2012). Furthermore, the lower doses included in many non-guideline studies are often more relevant to human exposure.

1.1.4 Impact of EDCs on the Mammary Gland

The effects of environmental chemicals on the mammary gland can be characterized into three major adverse effects: those that disrupt development, those that impair lactation, and those that induce cancers or increase susceptibility to breast cancer. Disruption can result in underdevelopment or overdevelopment of the gland. While many experimental studies have reported that EDCs induce changes to the gross structure of the mammary gland, often it is not the focus of studies as most regulatory and funding agencies are interested in carcinogenesis (Fenton, 2006). However, both the complexity of EDCs and the ability of EDCs to alter development of the mammary gland has been demonstrated by the case of bisphenol A (BPA). Initial studies examining the effects of BPA exposure on the rodent mammary gland observed reduced lateral and longitudinal growth in BPA-exposed animals compared to control groups. Yet, a follow-up repetition of the study reported no observed changes in exposed group (Nikaido et al., 2004). However, another study investigating low-dose specific effects of BPA found that BPA-exposed mice had signs of hypergrowth (Markey et al., 2001). Researchers observed increased and early onset of TEBs, increased lateral branching and complexity as the gland developed (Fenton, 2006). In BPA-exposed animals, as they aged, there was increased branching (Munoz-de-Toro et al., 2005), the abnormal presence of lobuloalveolar structures (Markey et al., 2003), and an increased risk of intraductal hyperplasias (Vandenberg et al., 2008), a pre-neoplastic lesion. Effects on the gross
morphology of the mammary gland can have further implications for lactation or susceptibility to carcinogenesis which will be discussed in the following sections. However, even the observation of over- or under-growth of the mammary gland is important as it indicates the exposure of interest altered development of the gland, which may occur due to disruptions of the hormones that regulate normal mammary gland growth (Soto et al., 2008).

Disruption of development and impaired lactation may not directly affect the individual; however, it impairs the ability to sustain normal growth of newborns (Marasco, 2014). Furthermore, maternal nursing has demonstrated to strengthen the immune system of the newborn as well as reduce risk of breast and ovarian cancer in the mother. Lactation can be impaired morphologically or in the endocrine dependent signaling pathways responsible for milk production. Additionally, there can be partial impairment of lactation resulting in insufficient milk supply and further the need for supplementary feeding. Unfortunately, breast milk has been demonstrated to accumulate certain environmental chemicals, allowing for direct fetal transfer of a compound during the lactation period (Anderson & Wolff, 2000). The effects of impaired lactation have been best exhibited in rodent studies of PFOS (White et al., 2009, Fenton et al., 2009, Reiner et al., 2009). Researchers found that despite no changes to mating or fertility, there were multiple notable lactation outcomes (Fenton et al., 2009). First, there was reduced milk supply in dams leading to decreased body weight and altered growth trajectories in pups (Fenton et al., 2009). Researchers also observed higher rates of pup deaths during lactation among PFOS exposure groups. Researchers proposed that this
could be due to either the reduced nutrition or perhaps direct exposure to PFOS that accumulates in breast milk (White et al., 2009).

In another study conducted on the histological changes of the mammary gland in response to various EDCs found in personal care products, researchers observed multiple changes to the mammary gland structure that could have implications for lactation (Manservisi et al., 2015). The compounds of interest were diethylphthalate (DEP), methylparaben (MPB), and triclosan (TCS), and a mixture of all three (MIX). Primarily, animals treated with any of the test chemicals or MIX had unfilled alveoli (i.e., lactational structures lacking milk) compared to the controls. This observation was in addition to an increase in adipose tissue as well as the observation of collapsed alveolar structures or ducts that showed residual secretory content. Researchers further investigated the whole mount samples from these DEP/MBP/TCS/MIX-exposed mice and found corroborating morphologic patterns of impairment. Observations included disparities in lobular development, with animals exposed to all compounds demonstrating fewer but denser lobular structures. In the TCS-exposed females, there was an obvious reduction in size and number of lobular structures and histological analysis revealed a lower number of alveoli, most of which were empty during the lactation period (Manservisi et al., 2015).

Carcinogens are compounds that have the ability or tendency to promote the formation of cancer (Searle, 1984). However, it is important to not just identify chemicals that are direct carcinogens, but also those that also increase the gland’s susceptibility to cancer. Numerous studies have demonstrated associations between EDC exposures and cancer risk, however the exact mechanisms by which these EDCs act are
still unclear, because most are not direct carcinogens and most are not genotoxic (Soto et al., 2013). DES is one of the most common examples of an EDCs that has been studied for its carcinogenicity (McLachlan, 2006). Strong epidemiological evidence has been produced from a cohort of women gestationally exposed to DES (Newbold, 2004). Women exposed to DES during fetal development have significantly increased rates of a rare vaginal cancer that has since been almost solely associated with DES exposure (Newbold & McLachlan, 1996, Hatch et al., 2001). Furthermore, as this cohort ages, there is increasing evidence of increased breast cancer risk among those gestationally exposed to DES (Palmer et al., 2006).

Dioxins are another EDC that has been shown in animal models to increase susceptibility to chemical carcinogens (Shwarz & Appel, 2005). Rats exposed to dioxins developed increased numbers and severity of tumors when challenged with known carcinogens. Morphological changes and prolonged retention of TEBs were observed in histopathological analysis of dioxin-exposed rats. Epithelial tissue is the most common site for cancer development, thus researchers deduced that the increased susceptibility may be due in part to the increased prevalence of epithelial structures in exposed animals.

Many researchers agree that there is a role for both developmental disruptions as well as later in life alterations that can result in increased susceptibility and/or carcinogenesis. One major theory is that cancer is due to an accumulation of genetic mutations in the cell (some of which may be influenced by hormonal disruption) (Schug et al., 2011). A second theory is that exogenous hormones can cause integral changes to the epigenome that increase carcinogenesis and susceptibility later in life (Schug et al., 2011).
Regardless of the exact mechanism, both theories propose that cancer is a result of dysregulated cell function and proliferation. Thus, research on the effects of carcinogen exposure during in utero development is critical to understanding factors that may contribute to later in life disease.

### 1.1.5 The mouse Mammary Gland: A model of mammary gland development

Mice and rats make good models to understand growth and development of the human breast due to similarities in structure, development, and hormonal regulation between the species (J.G. Vandenbergh 2004). Of course, each model has its strengths and deficits, and researchers may choose one species over another based on experimental factors or features of the toxicant being evaluated. This experiment utilizes a mouse model which has been previously used to evaluate the effects of environmental estrogens on development. Mice have a shorter lifespan and larger litter size which increases the statistical power of analysis (J.G. Vandenbergh 2004, Stokes, 2022). Additionally, the development and function of the mouse mammary gland has been well characterized due to the development of numerous genetically modified strains (Manibusan & Touart, 2017). Through the use of knockout models and in vitro analysis, researchers have elucidated the role that numerous hormone signaling pathways play in the development and function of the mammary gland (Patisaul, Fenton & Aylor 2016).

There are important differences between the mouse and human mammary gland including the percentage of the gland comprised of connective tissue rather than adipose, and the differentiation of lobular structures in non-pregnant adults (Hovey, McFadden, and Akers, 1999). Yet, researchers have found that despite differing tissue compositions, the murine and human mammary gland still have similar paces of development (J.G.}
Vandenbergh 2004, Stokes, 2022) and are similarly sensitive and dependent on estrogen (and other growth hormone) signaling (Hovey, McFadden, and Akers, 1999). Finally, transcriptome analyses have revealed numerous conserved genes relating to cell-fate and differentiation between the murine and human mammary gland.

Although there are some important structural differences between the mammary gland of rodents and humans, these species have similar developmental trajectories and similar roles for ovarian and pituitary hormones; thus, the rodent is often used to model how chemicals, including many EDCs, may affect human development (De Cicco, et al. 2011). The main structural difference between the mouse and human mammary gland is that in mice one ductal tree fills the fat pad stemming from the nipple. In humans however, there are multiple ductal trees that join at the nipple (McNally & Stein 2017). Another structural difference between mouse and human mammary glands is in the budding structures that begin to appear at the end of puberty. In mouse anatomy we observe small numbers of alveolar buds that form with each round of the estrous cycle, whereas in humans we see the growth of terminal duct lobular units. Comparable units are not induced until the late stages of pregnancy in mice (McNally & Stein 2017). Finally, another key difference to consider is the difference in estrous cyclicity between mice and humans and how that may influence the hormonal milieu at a given point in time (McNally & Stein 2017). Despite these specific structural differences, generally the type of tissue, timing of development, and influence of hormonal signaling is largely similar between mice and humans. When studying the mammary gland in a rodent model, it is important to understand the key similarities and differences between different animal models and human mammary gland function to best interpret laboratory findings. In both
humans and rodents, the mammary gland develops predominantly postnatally. Mice are a particularly good model because transgenic and gene knockouts have been created and studied over the past two decades. These studies have helped to inform knowledge of the role that steroid hormones and hormone receptors play in the development of the gland. Additionally, studies suggest that these components have similar mechanisms of action and biological pathways across species (McNally & Stein 2017).

The rat model has its own benefits, and thus this species may also be appropriate for studies of environmental pollutants. Most rat strains have a greater susceptibility to carcinogenesis compared to mice; the rat gland also has more differentiating lobular structures that makes it morphologically more similar to the human than the mouse (Medina, 2007, Cardiff & Wellings, 1999). However, the rat has a much more complex developing gland, which makes it a more challenging model to utilize for studies evaluating the effects of environmental chemicals on development and morphology.

1.1.5.2 Techniques to assess the effects of environmental pollutants on the rodent mammary gland

Unfortunately, as described in more detail below, typical assessments of mammary glands in standard toxicity tests used for regulatory purposes often overlook the mammary gland or use methods that are insufficient to characterize the effects of environmental chemicals. Studies developed by the Organization for Economic Cooperation and Development (OECD) are often used as regulatory standards. Key guidelines for evaluating developmental and reproductive toxicity currently lack mammary gland analysis. For example, test guideline (TG) 421 which evaluates developmental and reproductive toxicants does not mention the mammary gland
anywhere in the guideline. The lenient guidelines of TG 443 (to be discussed in later chapters), only notes the option to collect the mammary gland if previous research indicates potential effects. Furthermore, the recommended analysis is simply “microscopic analysis”, with additional information in guidance documents that is similarly vague. Yet, numerous laboratories with expertise in the mammary gland have developed complementary methods to evaluate different aspects of mammary gland biology (Makris 2011). In these labs, current methods of analysis include characterization of whole mounted mammary glands for morphological assessment of the entire gland. These glands are often imaged with a dissection microscope and the mammary epithelial structures are measured for size and the abundance of epithelial structures (e.g., branching points, terminal ducts, terminal end buds, etc.). Changes in size or number of specific structures or evaluation of the gland as a whole can indicate different effects on the mammary gland. For example, branching points can be utilized to quantify a measure of ductal complexity. Additionally, the evaluation of terminal ducts (sometimes called terminal ends) can indicate the termination of growth allowing researchers to evaluate timing of mammary gland development. Finally, TEBs can also indicate normal or abnormal ductal extension. However, prolonged or abnormal presence of TEBs may also indicate an increased susceptibility to carcinogenic insults. These preliminary quantifiable measurements are essential to guiding further analyses and can give insight into morphological changes in the organ even when the exact biological mechanism remains unknown.

Other approaches to evaluate the mammary gland use sectioned tissue, collected longitudinally to ensure mammary ducts are observed across the regions of the gland, for
histopathological and immunohistochemical analyses. Frozen tissues can also be used for gene expression analysis. These methods of evaluation can identify and represent hormone receptor expression, cellular proliferation, morphological changes or abnormalities, as well as alterations to gene expression.

Previous studies assessing the effects of EDCs and the mammary gland have reported that developmental exposures to estrogenic chemicals can affect the long-term health of the mammary gland (Macon & Fenton 2013). Some of the changes that have been documented include delayed development (e.g., a reduction in the size of the ductal network or number of TEBs at a specific developmental stage), ductal hyperplasia, alveolar hypoplasia, reduced apoptosis in TEBs, altered gene and protein expression, changes in the number of terminal ducts, and accelerated alveolar differentiation (De Cicco, et al. 2011). The effects of these compounds are dependent on the timing of exposure, timing of evaluation, dose, and other chemical-specific features. Yet, many of these effects are sufficiently consistent that researchers have proposed that the rodent can be used to predict adverse health outcomes that have been documented in human populations (Rudel et al. 2007).

1.1.6 Phthalates

Phthalates are a class of compounds used in numerous everyday products. They are classified by their chemical structure which consists of one benzene ring and two ester groups; they are differentiated by their ester groups and the length of the ester’s carbon chains (Ventricе, et al. 2013). The most common use of phthalates is as plasticizers, e.g., additives included in plastics that increase the material’s properties such as flexibility and durability. Plasticizing phthalates are used in PVC products, wiring, flooring, fabrics, and
even food packaging. Some of the lower molecular weight phthalates are also used in fragrances, cosmetics, and personal care products (Katsikantami, et al. 2016).

Human exposure to phthalates can occur through ingestion, inhalation, and dermal exposure. Ingestion of phthalates occurs by eating contaminated food, which absorbs chemicals from food packaging as well as food processing and manufacturing equipment (Katsikantami, et al. 2016). It is a common misconception that phthalates only leach into food products under specific conditions (e.g., high temperatures associated with sterilization of food packaging). However, even at ambient temperatures phthalates can be released from plastic materials and migrate to food products (Ventricre, et al. 2013). Thus, even when products are utilized “as intended”, unintended human exposures to phthalates occur.

Beyond their use in food packaging and food production, human exposures occur via non-oral routes of exposure. Inhalation of phthalates can occur via indoor air pollution when phthalates are used and then released from housing materials such as PVC vinyl flooring (Katsikantami, et al. 2016, Kang et al., 2005). Phthalate exposures can also occur due to dermal absorption when they come in direct contact with the skin, for example through contact with personal care products (Ventricre, et al. 2013). While phthalates can be found in synthetic fabrics, researchers are still investigating whether these compounds can be absorbed into the skin directly from such products, or if the fabrics could provide protection for bare skin from other exposures (Katsikantami, et al. 2016).

When phthalates enter the human body, they are often metabolized into their monoesters, allowing excretion of their more water-soluble products. Some phthalates are more readily metabolized and excreted than others and the time to excretion is dependent
on the ester groups (Katsikantami, et al. 2016). Metabolism of phthalates normally occurs in two phases. The first phase of metabolism is hydrolysis into the primary monoester phthalate. This process utilizes lipases and esterase in the intestine and parenchyma to catalyze this phase I metabolism (Frederiksen et al, 2007). Some monoester phthalates are excreted through urine or feces after this first phase of metabolism while others must go through several biotransformations such as additional hydroxylation or oxidation (Frederiksen et al, 2007). However, other phthalates go through a phase two metabolism. Generally, this process is catalyzed by uridine 5’-diphosphogluconuronyl transferase (Frederiksen et al, 2007). Once conjugated with this enzyme, the phthalate forms a hydrophilic glucuronide conjugate which is then excreted in urine (Frederiksen et al, 2007). This process becomes increasingly complex as new phthalates are produced. Often phthalates can have numerous metabolites that may have different biological activity compared to the parent compounds.

Numerous studies have identified several key vulnerable populations for phthalate exposure. Pregnant women and children are two such populations for a number of reasons: 1) metabolites of phthalates are distributed by the circulatory system and they often accumulate in breast milk and amniotic fluid, leading to higher exposures during early life stages (Huang et al. 2009) exposures to some phthalates can be higher due to the increased rate of respiration, food intake, and water intake during these life stages; 3) numerous organs are developing, and cells are differentiating during these life stages, making them more susceptible to environmental insults; 4) processes of development and differentiation in these populations are tightly linked to hormonal regulation which can be disrupted by phthalates as discussed in more detail below.
Phthalates have been identified as EDCs because of their ability to block the synthesis of androgens; some also act as androgen receptor antagonists, while others have estrogen receptor agonist properties (Ventric, et al. 2013). Phthalates have also been classified as developmental and reproductive toxicants in numerous animal studies. Specifically, a prospective cohort study conducted in Taiwan found that fetal exposures (as measured in maternal urine and umbilical cord samples) to several phthalates were associated with adverse health outcomes in newborns. Researchers involved in this study concluded that gestational phthalate exposures influenced hormone signaling pathways, fertility, and sexual differentiation (Huang et. al. 2009).

1.2 Butyl Benzyl Phthalate

Butyl Benzyl Phthalate (BBP) is a lightweight phthalate with the appearance of a clear liquid at room temperature (Staples et al., 1997). Because of the chemical structure of the compound, BBP can easily leach through soil into ground and drinking water (Staples et al., 1997). BBP is found commonly in products made with PVC including vinyl flooring, pipes, carpet backing, and even food packaging (Tyl et al., 2004). Adults are estimated to be exposed to 2 μg/kg body weight per day whereas young children, and especially infants, could be exposed to two to three times that amount (Ahmad et al., 2013). The most common route of exposure to BBP is ingestion of contaminated foods, water, and dust particles (Tyl et al., 2004).

While some phthalates (like di (2-ethylhexyl) phthalate or DEHP) have been studied extensively in animal models, research on the effects of BBP exposure is limited and inconsistent. In the United States, the National Toxicology Program’s Center for the Evaluation of Risks to Human Reproduction (CERHR) investigated BBP due to its
widespread detection in the environment. This report, completed by a contract laboratory, concluded that there was negligible concern for human health because adverse effects in rodents were only observed after high levels of exposure, beyond those typically experienced by humans (Tyl et al., 2004).

While BBP is considered a low priority toxicant in the United States, it has been strictly regulated and even banned in children’s toys by the European Commission since 1999 (Pakalin et al, 2008). This regulation against BBP use in the European jurisdiction was based on the hazard data that are available, its classification as an EDC, and the legal application of the precautionary principle. In their decision, the European Commission determined that there is sufficient documentation of developmental effects in animal studies that BBP should be avoided.

1.3 BBP is an EDC: Evidence of Mechanism of action

Signaling via the estrogen receptor (ER), and other nuclear hormone receptors, plays crucial roles in sexual maturation and reproduction. There are two subtypes of the nuclear ERs, ERα and ERβ. These two receptors are members of the same family; however, they have different expression levels and divergent functions in tissues. For example, ERα is central to the development of the mammary gland, with the ERα knockout mouse having a stunted mammary gland morphology that is incapable of producing sufficient milk for offspring (Couse, 1999). Mice lacking the ERα gene had normal gestational development of the mammary gland structure but failed to grow and proliferate during the subsequent essential windows of development later in life (puberty and pregnancy/lactation) (Couse, 1999). In contrast, female mice lacking ERβ have a normal mammary gland morphology throughout puberty and early adulthood, but some minor deficiencies that manifest during
pregnancy and lactation (Couse, 1999). However more current research indicates a more significant regulatory role of ERβ in the mammary gland. In a new knockout model, researchers have found that mice without ERβ had epithelium that was not fully differentiated and had changes in the levels of adhesive molecules (Warner et al., 2020). Mice lacking ERβ showed overexpression of ERα in addition to epithelial overgrowth (Warner et al., 2020). The emerging research on ERβ is highlighting the receptor for its importance for regulation of differentiation and epithelial growth via modulation of the ERα (Warner et al., 2020). While ERβ itself may not have a direct role in estrogen signaling in the gland, its importance to normal ERα function makes it just as essential to the mammary gland and reproductive development as whole.

Predominantly, research has focused on the nuclear ERα (Takeuchi et al., 2005). ERα is localized to the cytoplasm until it is activated by binding of the ligand (i.e., 17β-estradiol). Binding of the steroid activates the complex via conformational change which in turn promotes ERα homodimerization and gives the complex a high affinity for DNA (Hall et al., 2002). Because of this, once ligand-bound, the receptor complex acts as a transcription factor; the bound receptor migrates into the nucleus, binds to specific locations on the DNA known as estrogen-responsive elements (ERE), and regulates the expression of various down-stream genes (Hall et al., 2002). The key difference between the different ERs is that while an active ERα undergoes homodimerization, ERβ undergoes heterodimerization where it binds to ERα before it acts on DNA and gene expression.

An ER agonist is a molecule that binds to and activates the ER. This could be a naturally occurring estrogen molecule such as 17β-estradiol, or one of the other
endogenous estrogens such as estrone and estriol. Other ER agonists include naturally occurring phytoestrogens such as genistein and genistin, and naturally occurring mycoestrogens such as zearalenone. Finally, ER agonists include xenoestrogens, exogenous compounds with estrogen mimicking properties (Wood et al., 2022).

1.3.1 Evidence that BBP is an Estrogen Receptor (ER) agonist

There is evidence from several different studies that BBP is an ER agonist. One study conducted in 2005 examined 22 phthalates for ER agonist activity utilizing a transactivation assay with human hormone receptors. In this assay, cells are transfected with luciferase plasmids tailored to detect ER-binding activity; once the ER is bound (by either endogenous or exogenous ligands), dimerized ER binds to a transgene and expression of luciferase is induced. This expression can be quantified using a plate reader with a spectrophotometer and compared to a standard curve, allowing the estrogenic effects to be compared to a positive control (like estradiol). In the study of phthalates, of the 22 phthalates evaluated, BBP (referred to as BBeP in this specific study) demonstrated the greatest estrogenic activity in an assay evaluating ERα. BBP was additionally the only phthalate among the 22 that activated ERβ (Takeuchi et al., 2005).

Similar results were also found in a yeast based estrogenicity assay (Kang & Lee 2005). In this study, a yeast-based ER gene transcription assay was utilized to evaluate estrogenicity of multiple phthalates (Kang & Lee 2005). Researchers then evaluated methylation of genes using multiple cell lines following exposure to phthalates. Methylation status was evaluated via methylation-specific PCR analysis and multiple known estrogenic compounds were included as positive controls for comparison. Effects occurring via ERα were evaluated by quantifying the expression of a proxy reporter, i.e.,
using beta-galactosidase activity. BBP was found to be weakly estrogenic. However, its monoester metabolites all lacked estrogenic potential. Methylation analysis revealed that BBP exposure was associated with demethylation of the ERα promoter and increased expression of ERα.

One reason why the estrogenic properties of BBP have caused some confusion is that other studies have revealed that it is not just an ER agonist, it is also an ER antagonist. Studies with MVLN cells revealed both ER agonist and ER antagonist activities, depending on the concentration of BBP that was evaluated (Ghisari & Benefeld-Jorgensen, 2009). This study used an ER transactivational assay with a luciferase reporter to evaluate the estrogenic effects of BBP (among other phthalates) on ERα and estrogen-mediated pathways. BBP was shown to weakly, but significantly, induce luciferase activity, consistent with an ER agonist. However, at higher concentrations, when BBP and estradiol were co-administered, BBP inhibited the estradiol-mediated responses, consistent with an ER antagonist (and anti-estrogenic activity).

There is also evidence that some of the effects of BBP on estrogenic endpoints are not due to direct interaction of the phthalate with the receptor (as an agonist or an antagonist), but rather that BBP alters the expression of the receptor and that BBP alters the production of estrogens in target cells. For example, as previously stated, studies with MCF-7 cells revealed that low concentrations of BBP induce demethylation of the ERα transcript, which is associated with induced expression of the receptor (Kang & Lee 2005). Another study aimed to evaluate antiestrogenic and antiandrogenic activity of commonly used phthalates via a XenoScreen YES/YAS assay (Czernych et al, 2017). Researchers found a slight increase in beta-galactosidase synthesis in the estrogenicity
test; however, the effects were small and insignificant. Researchers in the same study noted significant anti-estrogenic activity however, and upon further research concluded that BBP exposure could inhibit receptor-mediated estradiol production.

1.3.2 Effects of BBP on Androgen Receptor (AR) signaling

In vitro studies have also revealed that BBP has anti-androgenic effects. Androgens are naturally occurring steroid hormones whose main function is development and maintenance of male characteristics and sexual function. In males, androgens acting via binding to ARs are essential to embryonic, fetal and pubertal development and adult reproductive functions including spermatogenesis. Epidemiological and animal model studies have associated androgen dysregulation with birth defects including decreased anogenital distance, undescended testes (a condition called cryptorchidism), and abnormal placement of the urethral opening on the penis (a condition called hypospadias). While androgens are often considered the male sex hormone, they have integral functions in bodily pathways in both males and females (Sohoni & Sumpter, 1998). For example, androgens play important roles in fat deposition, muscle mass and development, as well as brain function and development. Androgens also have a unique role in female pregnancy as they are integral to controlling uterine contractions and preventing preterm labor. Finally, androgens are the precursors to estrogens; aromatase, a cytochrome P450 enzyme expressed in many of the body’s tissues, converts androgens to estrogens.

AR is a ligand-dependent transcription factor and dihydrotestosterone (DHT) is a potent endogenous ligand. Like the ER, binding of a ligand to the AR activates its transcription regulatory functions. Numerous AR antagonists can disrupt the actions of
endogenous androgens by either binding to the receptor binding pocket, preventing the binding of ligands such as DHT or testosterone, or by binding elsewhere on the receptor, changing the conformation of the receptor and preventing the endogenous ligand from binding. Additionally, anti-androgenic compounds have been proposed to create an “estrogenic environment” thus yielding effects similar to those of an estrogenic compound (Sohoni & Sumpter, 1998).

The androgenic and anti-androgenic potential of multiple xenobiotics was evaluated using a yeast screening assay (Sohini & Sumpter, 1998). Multiple EDCs including BBP were evaluated using yeast that were transfected to express the human AR. BBP demonstrated no direct androgenic effects however it successfully inhibited the activity of DHT in a dose dependent manner, consistent with an AR antagonist (Sohoni & Sumpter, 1998).

The anti-androgenic potential of BBP was also observed using another yeast reporter (the XenoScreen YES/YAS in vitro assay) (Czernych et al., 2017). Again, researchers found that BBP demonstrated no significant AR agonistic properties. However, BBP showed significant inhibition of DHT, consistent with an AR antagonist. Furthermore, BBP was an efficient AR antagonist, with 93% inhibition of the action of DHT.

Despite some compelling anti-androgenic effects, the AR-mediated effects of BBP are varying. While a few studies demonstrate anti-androgenic activity of BBP (Takeuchi et al., 2005), others report no effect (Roy et al., 2004 & Sultan et al. 2001). The variation in evidence among different cell lines indicates that observed/unobserved AR-mediated effects could be due to a technical effect (e.g., differing sensitivity or reliability between
assays), or a biological effect (e.g., differences in co-factor expression between cell types).

1.4 BBP is an EDC: Evidence from laboratory animal studies

AR signaling plays a key role in spermiogenesis making it pivotal to male reproductive capacity (Davey & Grossman, 2016). Like many other phthalates, studies in laboratory animals revealed that BBP induces developmental and reproductive effects when animals are exposed during gestation (Moral, et al. 2007. BBP exposures during early development are associated with decreased anogenital distance, undescended testes, and cleft phallus in exposed male rodents (Tyl et al, 2004). Other studies have reported additional adverse effects of BBP exposure on the development of the male reproductive system including decreased sperm count and malformation of accessory sex glands (Ahmad, et al. 2013, Gray Jr. et al, 2000, Tyl et al., 2004).

Results in females exposed to BBP during development are more ambiguous and there is less congruence between studies. One study examined the effects of BBP on progesterone-mediated outcomes (Funabashi, Kawaguchi & Kimura 2001). Progesterone receptors (PR) are a part of the nuclear hormone receptor family and integral to mammary gland development as well as reproduction. BBP exposures in rodents increase serum levels of progesterone (Picard et al., 2001) and a single dose of BBP in adult female rats was sufficient to significantly increase the expression of PR mRNA in the preoptic area of the hypothalamus (Funabashi, Kawaguchi & Kimura, 2001). These findings are noteworthy as PR signaling is involved in facilitating female reproductive functions such as hormone secretion and lordosis behavior. However, there is very little
literature exploring the mechanisms behind these observed effects, or whether they are associated with adverse outcomes.

1.4.1 The effect of BBP on the rodent mammary gland

A small number of studies have focused on understanding the effects of BBP exposures on the rat mammary gland. The first conducted an in-depth analysis of the effects of BBP (among other EDCs) on the genomic profile of the rat mammary gland following developmental exposures (e.g., exposures during the prenatal period) (Moral, et al. 2007). Outcomes were assessed at several postnatal time points before, during, and after puberty (postnatal days [PND] 21, 35, 50 and 100). Most effects of gestational BBP exposure were detected in the pre-pubertal and pubertal onset period of the rat lifecycle between PND 30 and 50. Gene expression analyses using microarrays revealed 515 upregulated genes in BBP-exposed mammary glands at PND21. Function analysis of these differentially expressed genes found that proliferation and differentiation, signal transduction, and cell communication pathways were significantly overrepresented; 141 are hypothesized to play a role in tissue morphogenesis and cell differentiation, transcription factors, cell proliferation, stress response, signal transduction, metabolism, transport and cell organization. Only one gene was downregulated in the PND21 mammary gland (gad1). The analysis of the genomic profile of BBP-exposed rats revealed the importance of timing for the evaluation of the mammary gland (Moral, et al. 2007). Surprisingly, at PND35 only 6 genes were differentially expressed in the BBP-treated females. At PND50, however, this number had increased to 25 genes that were upregulated and 14 that were downregulated. Functional analysis at this time point (e.g.,
during the transition from late puberty to early adulthood) indicates an over-representation of downregulated genes coding for cytoskeletal proteins.

While their 2007 study did not indicate any significant morphological changes in the mammary gland, increased proliferation that was reported in the rat mammary gland (Moral, et al. 2007) warranted further investigation. In a follow-up study (Moral et al., 2011), changes to mammary gland morphology and the proliferative index of mammary epithelial cells were observed well after treatment had ended, with effects that were significant at PND35. This skewed ratio of cell proliferation/apoptosis can increase TEB susceptibility to malignant transformation. Importantly, the highest proliferation indexes were observed in BBP-treated females at PND35, compared to the controls which had the highest proliferation at PND50. As this timing is indicative of the period with greatest susceptibility to carcinogenesis, these results may indicate a shift in the window of vulnerability to other environmental stressors including chemical carcinogens. These outcomes were also supported by gene expression analyses, which revealed that multiple genes related to apoptosis were downregulated in BBP-treated mammary glands whereas Cryab, a gene with anti-apoptotic effects, was upregulated. Long lasting effects were observed in functional groups of genes related to immune function, cell signaling, proliferation, and differentiation. BBP also was found to alter expression of genes related to DNA damage repair at multiple life stages and doses of exposure.

1.5 The Extended One-Generation Reproductive Toxicity Test (TG 443)

The Organization for Economic Co-operation and Development (OECD) is an international organization with the overall goal of shaping policies that promote the betterment of all people. It is a collaborative effort from 35 countries and the European
Union to create a foundational forum for exchanging research and policy experiences. The OECD has established evidence-based research standards to promote comparable and valid research across many topics and disciplines including social, economic, and environmental challenges (OECD 2018).

The evaluation of environmental chemicals is a topic of high interest, and Test Guidelines (TG) have been established by the OECD to assess chemicals using standardized methods (OECD 2018). However, before the implementation of a guideline, it must first be developed, validated, then accepted by the OECD as a regulatory standard. The OECD takes precautions to produce the most comprehensive guidelines with supporting documents for experimenters. OECD guidelines are often developed by a multifaceted team of experts who draw from existing scientific methods, regulatory practices, and good laboratory practice standards established by the OECD. Many OECD guidelines build from existing guidelines or incorporate already established guidelines.

The second component to guideline development is validation of the study design. There is a foundational set of principles first described in 1996 known as the “Solna Principles” that should frame all validation studies. In a recent document further describing test method validation, the OECD published and updated the Solna Principles to reflect the progression of the OECD. These updated principles and criteria for test method validation are as follows (note that the text below comes directly from the OECD’s documentation):

\( a) \) The rationale for the test method should be available. This should include a clear statement of the scientific basis, regulatory purpose and need for the test.

\( b) \) The relationship between the test method's endpoint(s) and the (biological) phenomenon of interest should be described. This should include a reference to scientific relevance of the effect(s) measured by the test method in terms of their mechanistic (biological) or empirical (correlative) relationship to
the specific type of effect/toxicity of interest. Although the relationship may be mechanistic or
correlative, test methods with biological relevance to the effect/toxicity being evaluated are preferred.
c) A detailed protocol for the test method should be available. The protocol should be sufficiently
detailed and should include, e.g., a description of the materials needed, such as specific cell types or
construct or animal species that could be used for the test (if applicable), a description of what is
measured and how it is measured, a description of how data will be analyzed, decision criteria for
evaluation of data and what are the criteria for acceptable test performance.
d) The intra-, and inter-laboratory reproducibility of the test method should be demonstrated. Data
should be available revealing the level of reproducibility and variability within and among
laboratories over time. The degree to which biological variability affects the test method
reproducibility should be addressed.
e) Demonstration of the test method’s performance should be based on the testing of reference
chemicals representative of the types of substances for which the test method will be used. A sufficient
number of the reference chemicals should have been tested under code to exclude bias (see paragraphs
on “Coding and Distribution of Test Samples”).
f) The performance of the test method should have been evaluated in relation to relevant information
from the species of concern, and existing relevant toxicity testing data. In the case of a substitute test
method adequate data should be available to permit a reliable analysis of the performance and
comparability of the proposed substitute test method with that of the test it is designed to replace.
g) Ideally, all data supporting the validity of a test method should have been obtained in accordance
with the principles of GLP. Aspects of data collection not performed according to GLP should be
clearly identified and their potential impact on the validation status of the test method should be
indicated.
h) All data supporting the assessment of the validity of the test method should be available for expert
review. The detailed test method protocol should be readily available and in the public domain. The
data supporting the validity of the test method should be organized and easily accessible to allow for
independent review(s), as appropriate. The test method description should be sufficiently detailed to
permit an independent laboratory to follow the procedures and generate equivalent data. Benchmarks
should be available by which an independent laboratory can itself assess its proper adherence to the
protocol.

1.5.1 OECD Work on Endocrine Disrupting Chemicals

The OECD has established EDCs to be a topic of interest in international research and
collaboration. The overall goal of the OECD is to help identify EDCs and their effects in
both human and animal populations. The development of EDC evaluation test methods is
focused largely on the effects of chemicals that interfere with (including chemicals that
mimic) the actions of estrogens, androgens, and thyroid hormones. There is no single
approach that is used to identify, prioritize, or characterize chemicals that are EDCs.
Instead, the OECD has published a conceptual framework for testing and assessing
EDCs. Several TGs can contribute knowledge about the endocrine disrupting properties
of test chemicals. Information can be measured at varying biological levels such as
biochemical, cellular, organismal, and population levels.

The conceptual framework for testing EDCs therefore exists in 5 levels. Level 1 tests
are mammalian and non-mammalian toxicology testing on chemicals using already
existing data and non-test information. Level 2 studies are assessed via *in vitro* assays
providing data about specific endocrine mechanisms or pathways. Level 3 tests are *in
vivo* assays that provide data about selected endocrine mechanisms/pathways. Level 4
tests are *in vivo* assays which provide data on the adverse effects of endocrine relevant
endpoints. Finally, level 5 tests are *in vivo* assays which provide more comprehensive
data on the adverse effects on endocrine relevant endpoints over more extensive parts of
the organism’s life cycle. Examples of TGs utilized to identify or test chemicals to
determine if they are EDCs are listed below along with the level of the conceptual
framework that the TG is assigned to, and the hormonal pathway that is evaluated (Table
1.1).
Table 1.1 OECD test guidelines utilized to evaluate EDC’s.

<table>
<thead>
<tr>
<th>TG #</th>
<th>Name</th>
<th>Conceptual Framework Level</th>
<th>EDC evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>493</td>
<td>In Vitro Estrogen Binding Assay</td>
<td>2</td>
<td>Estrogen</td>
</tr>
<tr>
<td>455</td>
<td>In Vitro Estrogen Receptor Transactivation Assay</td>
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<td>Androgen</td>
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<td>H295R Steroidogenesis Assay</td>
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<td>Estrogen</td>
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<td>Androgen</td>
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<td>Fish Short-Term reproduction Test</td>
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<td>Estrogen, Androgen, and steroidogenesis</td>
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<tr>
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<td>Fish Screening Assay</td>
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<tr>
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<td>Amphibian Metamorphosis Assay</td>
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<td>Two Generation Reproduction Toxicity Study</td>
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1.6 Objectives of Following Chapters

Due to evidence that phthalates, and specifically BBP, demonstrate endocrine disrupting effects, and because prior studies have only evaluated a modest number of outcomes in female rodents, chapter 2 aims to assess the effects of gestational and perinatal exposure to BBP on the female mouse mammary gland. By exposing nursing dams with varying doses of BBP from pregnancy through lactation, the generation of interest is the F1 generation, which is only indirectly exposed. This thesis aims to quantify gross morphological changes in the mammary gland as well as alterations to epithelial cell proliferation and hormone receptor expression in the gland. Although this investigation of the mammary gland was not conducted as a part of an Extended One-Generation Reproductive Toxicity evaluation, it utilizes some of the methods that the Vandenberg lab and colleagues have suggested could be included in the EOGRT, including analysis of mammary gland morphology using the whole-mount gland.

Chapter 3 aims to evaluate the OECD test guideline 443: The Extended One-Generation Reproductive Toxicity guideline including a summary of studies conducted using TG 443. We determined which endpoints that are described in TG 443 were evaluated in each study as well as the outcomes that were reported.
CHAPTER 2

BUTYL BENZYL PHTHALATE AND THE ADULT MAMMARY GLAND

2.1 Introduction.

Phthalates are a class of plasticizers used ubiquitously in consumer goods and products that are found in virtually every aspect of today’s industrialized society. In spite of their use in many products, recent research has indicated a growing concern for adverse health outcomes associated with phthalate exposure. Butyl Benzyl Phthalate (BBP) is used in vinyl tile, carpet backing, products made with polyvinyl chloride including many other building products, children’s toys, and multiple other day to day plastic items. There are few epidemiological studies examining exposures to BBP and health outcomes, whereas controlled studies conducted in laboratory settings have produced significant but sometimes contradictory findings. Laboratory evidence of the endocrine disrupting potential of BBP was considered sufficient for it to be banned in toys and childcare items by the European Union (Pakalin et al, 2008). In contrast, the US National Toxicology Program Center for the Evaluation of Risks to Human Reproduction has deemed BBP of minor concern for adverse reproductive effects (although the expert opinion notes the need for further research into its effects on female reproductive outcomes).

A study conducted in 2008 characterized the metabolism of BBP using isotope labelling (Li et al., 2010). They investigated two routes of exposure, intravenous and oral, using administration of two doses, 10mg/kg and 100 mg/kg. Researchers administered the labelled BBP to determine its major metabolites, half-life, absorption, distribution, and elimination in the mouse model for both routes of exposure. It should be noted that
this was a singular exposure for the purpose of producing toxicokinetic data, but then has a limitation of not being able to evaluate whether toxicokinetic profiles would be altered by repeated exposures. By collecting blood samples over the course of two hours post-administration, the researchers conducting this study concluded that there is a high rate of absorption of BBP after oral exposure, with a Tmax, the time when blood concentrations are the highest, of 13 minutes. They additionally assessed tissues for the radioactive BBP, which gives a sense of its distribution (or the distribution of its metabolites, which would retain the radioactive marker) to the body tissues. Following exposure via intravenous injection, there was the largest distribution in (decreasing order of detection) the intestines, prostate, liver, lungs, fat, thymus, kidney, serum, testes, pancreas, spleen, muscle, heart, bones, stomach, and brain and evaluated urine and feces for excretion. The intestines, lungs, fat, kidney, prostate, liver and thymus had higher concentrations in the organ than in the serum. However, when investigating the oral route of exposure, researchers found the highest amounts of BBP in the liver, serum, kidney, lung, heart, muscle and brain.

Excretion of a compound is most commonly in urine or feces but can also occur in breast milk, sweat, skin, or respiration; most phthalates are excreted in urine in humans but can be excreted in both urine and feces in rodents. Following the mice that had been administered radiolabeled BBP, 4 days after exposure the mouse had excreted 80% of the original applied dose via urine and feces (Li et al., 2010). However, the researchers found that the main excretory route greatly depended on the route of exposure. Oral ingestion of BBP leads to excretion that is predominately via feces. This is compared to injected animals which largely excreted BBP metabolites in urine. Combining all of this data,
researchers could determine that BBP has a fast absorption rate, is quickly distributed into tissues and organs, but then more slowly eliminated from the body. The results indicate that the large intestine is likely the major site for metabolism of BBP, but the reproductive organs and the liver are likely the target organs of BBP. Prolonged exposure or retention of BBP in these tissues could have adverse effects on the animal (Li et al., 2010).

Laboratory studies of BBP in rodents and cell lines have indicated that BBP may act as an endocrine disrupting chemical. In male mice, gestational BBP exposure has effects consistent with an anti-androgenic mechanism of action. This is classified by the presence of alterations to the male reproductive system such as decreased anogenital distance and undescended testes (Moral et al., 2011; Tyl et al., 2004). These gross abnormalities are accompanied by observation of altered androgen receptor (AR) expression. In female rodents, while there is inconclusive evidence on the exact mechanisms of action of BBP, although most study results are consistent with an element of endocrine disruption.

A few studies have deemed BBP to have estrogenic effects via action on the ERα that were stronger than those observed for other known phthalates (Takeuchi et al., 2005; Kang & Lee 2005). Despite strong evidence of estrogenic effects, some studies concluded that the observed ER agonistic effects could be a consequence of BBP interaction elsewhere along the hormonal pathway rather than action solely as a receptor agonist. For example, one study proposed that BBP induces demethylation of the ERα transcript, inducing expression of the receptor itself (Kang & Lee 2005). Another study proposed that BBP may inhibit normal estradiol production, which can lead to downstream
alterations in the expression of ER expression in specific tissues, allowing those tissues to compensate for changes in circulating estradiol levels (Czernych et al., 2017).

Few studies have evaluated endocrine mediated effects of BBP beyond ER and AR signaling. Several studies suggest that BBP may have minor effects on thyroid hormone activity although to date these effects have not reach statistical significance (Ghisari & Bonefeld-Jorgensen, 2009, Li et al., 2020). Additionally, one study reported significant findings of BBP exposure on adrenal glands weight. This experiment was conducted on adult male rats which were exposed to low (50 mg/kg), mid (200 mg.kg), and high (1000 mg/kg) doses of BBP via corn oil ingestion once a day for 14 continuous days. Researchers observed that adrenal gland weights were reduced in the highest exposure group. However, the mechanisms responsible for these effects are not yet understood (Won Seo et al., 2004).

BBP may also affect progesterone signaling pathways. Progesterone receptor (PR) activity is closely linked to estrogen activity and multiple studies have observed that BBP exposures in rodents increase blood serum levels of progesterone (Picard et al., 2001, Funabashi et al., 2001, Ema et al., 1994). A single dose of BBP in adult female rats was sufficient to significantly increase the expression of PR mRNA in the preoptic area of the hypothalamus (Funabashi, Kawaguchi & Kimura, 2001). These findings are noteworthy as PR signaling is involved in facilitating female reproductive functions such as hormone secretion and lordosis behavior.

The mammary gland is particularly sensitive to estrogenic EDCs, making it an important tool to utilize in the evaluation of endocrine disrupting effects of BBP or its metabolites. A comprehensive study from the Russo group, described in depth in the
previous chapter, evaluated prolonged developmental exposure to BBP and mammary gland outcomes. After preliminary findings on altered gene expression (Moral et al., 2007), morphological alterations in the mammary gland were also described in BBP-exposed rats as well as changes to proliferation and differentiation in this organ. The researchers concluded that the seemingly disjointed results may point to an important shift in development in BBP-exposed rats, altering the window of susceptibility for the mammary gland (Moral et al., 2011).

This chapter focuses on better understanding the effects of BBP exposure by utilizing the sensitive mouse mammary gland. Evaluation of mice at three different doses and two ages allows us to observe outcomes during sensitive periods of mammary gland development.

2.2 Hypothesis

Because BBP has been shown to be an ER agonist, and because it has been shown to alter rat mammary gland morphology, we hypothesized that BBP exposures during perinatal development would alter mouse mammary gland morphology in puberty and adulthood. Specifically, we expected that BBP, like other ER agonists, would increase the number and size of TEBs in the pubertal gland and would increase epithelial structures including terminal ducts and alveolar buds in adulthood.

2.3 Methods

2.3.1 Animals & exposure

6–8-week-old BALB/c female mice were randomly assigned to one of four treatment groups. Females were then paired with males until mating occurred. Mice were housed and maintained in the University of Massachusetts Morrill animal facility in
polysulfone cages. They were given a standard chow diet and tap water from glass containers ad libitum. The facility was temperature controlled and lighting was on a 12hr light and dark cycle.

Beginning on the first day of pregnancy, determined by the presence of a sperm plug, dams were orally exposed daily to BBP. The compound was diluted in corn oil and administered with a pipette tip. Dams were administered the exposure through the end of lactation to ensure that the pups were exposed both in utero and throughout early development. The doses of BBP were selected to be relevant to human exposures. The lowest dose is the estimated 95th percentile of human exposure to BBP (3 µg/kg), followed by the tolerable daily intake dose (500 µg/kg). The final dose is representative of the NOAEL value established by toxicological studies (18 mg/kg). The control group received only tocopherol stripped corn oil for the same length of time as the exposure groups.

Treatment groups of the F0 generation dams included 8-12 animals per group. Dams delivered naturally and were allowed to nurse until weaning on postnatal day 21. Sex of each offspring was determined and recorded at weaning. After weaning, dams were moved to separate cages from offspring. To study the developmental effects of the F1 generation and avoid litter bias, one male and one female pup were selected from each dam for analysis at each age group (F1 puberty and F1 adults). Pups were cohoused with same-sex animals of the same treatment group and ear tagged for identification.

2.3.2 Euthanasia and Tissue Collection

Animals were euthanized at either puberty (PND 32-35) or in adulthood (postnatal week 9-11). Euthanasia occurred first with CO2 inhalation followed by
cervical dislocation to ensure death. The timing of euthanasia was not limited to specific stages of the estrous cycle. After euthanasia, we recorded the weight and anogenital distance of each animal. In male subjects, we collected the left and right fourth inguinal mammary glands. Male mammary gland evaluations are not reported in this thesis. From every female subject, we collected the right inguinal mammary gland for whole mount analysis and the left mammary gland for histology and immunohistochemistry.

2.3.3 Whole mount analysis

Whole mounted mammary glands were processed and stained according to standard laboratory protocols. The tissue was first processed through increasing grades of isopropanol (70%, 95%, and 100%) for one hour at a time. Following this step, samples were washed twice in toluene. The first was for one hour and the second was at least two hours in order to remove fat from the tissue. Samples were then rehydrated through decreasing grades of isopropanol (100%, 95%, and 70%) and then stained overnight using carmine. Carmine is utilized to distinguish ducts, epithelium, adipose cells, and stroma. In the final step of preparation, samples are dehydrated using a series of alcohols (70%, 95%, and 100%) and xylene for two hours. Stained samples were then sealed in k-pax heat sealed pouches with 5 ml of methyl-salicylate. Whole mounted and processed mammary glands were imaged using a Zeiss Axio Imager dissection microscope and Zeiss high-resolution color camera. Measurements were made using ZEN software.

Pubertal mammary glands were evaluated for ductal area, ductal extension, the number of terminal end buds (TEBs), and the area of TEBs. The adult whole mounted mammary gland is far more complex than pubertal structures, thus different morphometric measurement methods were utilized. At 30x magnification an image was
taken directly anterior to the central lymph node. We then overlayed a 15 by 13-point grid on the image and characterized the structures at each of the 195 crosshairs. Structures were classified as ducts, terminal ends, alveolar buds, blood vessel or stroma. Following counting of structures, the volume fraction of each was calculated by dividing the counted number of each structure by the total number of crosshairs.

2.3.4 Histopathology

Mammary gland tissues were fixed overnight in neutral buffered formalin and then washed in PBS, followed by dehydration in a series of alcohols. Samples were then embedded with paraffin under vacuum. Using a Fisher rotary microtome, samples were sectioned at 5 μm thickness and mounted on positively charged slides. Sectioned samples were then processed to stain the tissue. In brief, slides were deparaffinized in xylene and then rehydrated in series of increasing alcohol washes. Samples were then stained with hematoxylin, washed with acid alcohol, and counterstained with eosin. Samples then underwent another dehydrating process first with a series of alcohols, washed in xylene, then lastly, mounted with permount and a glass coverslip. Slides were imaged using an inverted microscope (Zeiss Observer Z1 inverted light microscope) with a 40X objective. Imaged cross section slides were overlaid with a 15 by 13 grid and characterized by the structures/tissue at each of the 195 crosshairs. Crosshairs were determined to be either stroma, ducts, alveolar buds, lymph node, or blood vessels.

2.3.5 Immunohistochemistry

Immunohistochemical analysis was conducted on sectioned adult paraffin samples. Expression of three markers was evaluated: ERα; Ki67, a marker of cell proliferation; and PR. In short, immunohistochemical analysis was conducted by
deparaffinizing the sections in xylene followed by a hydration process through a series of alcohols (70%, 95%, 100%). Next, samples were heated in a 10 mM citrate buffer and treated with hydrogen peroxide in order to quench endogenous peroxidases. Nonspecific binding sites were then blocked using a milk protein in 5% goat serum. Samples were then incubated with primary antibodies for 14-16 hours at 4C. After incubation, samples were treated with a secondary antibody and then a biotin complex. Samples were treated with diaminobenzidine chromogen in order to visualize immunohistochemistry results. Finally, hematoxylin was utilized to counterstain the images.

Evaluation of the processed slides consisted of taking non-overlapping images of each sample. 800 epithelial cells were evaluated for expression of the desired marker. Levels of ERα, Ki67, and PR were then produced as a percent ratio of the number of expressing cells to total epithelial cells.

2.3.6 Statistical analysis

Raw adult whole mount data were first utilized to determine the volume fraction of each of these structures: ducts, terminal ends, alveolar buds, and epithelial tissue by dividing the number of cross hairs characterized as each variable, divided by the total number of cross hairs. Pubertal whole mount data were continuous variable statistics that were analyzed without transformations.

First, descriptive statistics were calculated for each treatment including sample size, mean, standard error, and standard deviation. Following descriptive statistics, a one-way ANOVA was used to identify outcomes with significant differences based on treatment. Variables found to have significant statistical variance (P<0.05) were further evaluated using the Fisher’s least squared difference post hoc test with correction for
multiple comparisons. For data that were not normally distributed, non-parametric analysis was conducted. Hypothesis testing was conducted both via independent sample median tests, and a two-sided Kruskal Wallis Test.

Sections from adult female mammary glands stained with Hematoxylin and Eosin were evaluated using the same method of calculating the volume fraction of various ductal components. Volume fractions were then evaluated for descriptive statistics separated by treatment group. Descriptive statistics included sample size (N), mean, standard error, and standard deviation. Data were analyzed as described above.

Immunohistochemical data were evaluated to assess the percent positive cells by dividing the total number of positive cells per sample number by the total number of cells counted among all images of each sample. Descriptive statistics were then produced for % positive ki67, ER, and PR per each treatment group. This was followed by statistical analyses as described above. All statistical analyses were conducted in SPSS v28.

2.4 Results

2.4.1 Body weight

Body weight was recorded for both sexes, all treatment groups, and at both pubertal and adult timepoints. Average body weights for pubertal males (Figure 2.1A) increased in a monotonic dose-dependent manner, although this increase was not statistically significant (ANOVA, p=0.06). In the NOAEL group, the pubertal male body weight was 9% higher than the control males, an increase that may be considered biologically significant. Average adult male body weights for the control group, 95th percentile exposure group, TDI group and NOAEL group were not different between treatment groups (32.40 g, 31.50g, 32.5g, and 33.00g respectively; see Figure 2.1A). In
females, average pubertal body weight increased with higher BBP exposure. Specifically, females in the NOAEL group were significantly heavier than controls with a 9.9% increase in body weight (Figure 2.1B). Average adult female body weights also increased significantly in the BBP exposed females. The NOAEL group were 14% heavier compared to the control group. Average body weights were 24.63g, 26.33g, 26.14g, 28.05g for the control group, 95th percentile exposure group, TDI group and NOAEL group, respectively.
Figure 2.1. Average body weight (g) per treatment group. Treatment groups are control, 3 μg/kg (95th percentile), 500 μg/kg (Tolerable Daily Intake [TDI]), & 18000 μg/kg (NOAEL). (A) Average male bodyweights per treatment group at pubertal (Control N=7, 95th Percentile N=8, TDI N=9, NOAEL N=6) and adult (Control N=5, 95th Percentile N=6, TDI N=6, NOAEL N=6) time points with standard error. (B) Average female bodyweights at timepoints per treatment group. For female pubertal birthweight there was a significant difference between groups (p = .002). Following post hoc analysis, the 18000 μg/kg dose significantly varied from the lower dose groups: control (p* < .001), 95th percentile (p* = .003), TDI (p* = .004) P* < 0.05, fishers post hoc test.
2.4.2 Anogenital Index

Anogenital distance was recorded for both sexes, all treatment groups, and at both pubertal and adult timepoints. Pubertal females demonstrated a statistically significant change in anogenital distance ($p = 0.024$, data not shown). However, when AGI was calculated, accounting for differing body weight, there was no longer a significant difference based on treatment (Figure 2.2). In fact, there were no significant findings for male or female anogenital index between treatment groups at either puberty or adulthood (Figure 2.2).
Figure 2.2. Average anogenital index (AGI) per treatment group. Treatment groups are control, 3 \( \mu \text{g/kg} \) (95\textsuperscript{th} percentile), 500 \( \mu \text{g/kg} \) (Tolerable Daily Intake [TDI]), & 18000 \( \mu \text{g/kg} \) (NOAEL). (A) Average male AGI bodyweights per treatment group at pubertal (Control N=7, 95\textsuperscript{th} Percentile N=8, TDI N=9, NOAEL N=6) and adult (Control N=5, 95\textsuperscript{th} Percentile N=6, TDI N=6, NOAEL N=6) timepoints with standard error. No significant differences found between male groups at either pubertal or adult timepoints. (B) Average female AGI per treatment group at pubertal (Control N=6, 95\textsuperscript{th} Percentile N=7, TDI N=10, NOAEL N=10) and adult (Control N=8, 95\textsuperscript{th} Percentile N=9, TDI N=11, NOAEL N=11). No significant findings between groups at either time point.
2.4.3 Pubertal Female Whole Mount Mammary Gland Analysis

Pubertal female whole mount mammary glands were evaluated for ductal area, ductal extension, number of TEBs, total TEB area, average TEB size, and TEB density (Figure 2.3).

Whole mount analysis of pubertal females revealed no significant differences between treatment groups for any of the morphological parameters evaluated. However, it is noteworthy that both ductal area (ANOVA, p = 0.124) and ductal extension (ANOVA, p = 0.117) had outcomes more consistent with increased development in the higher BBP dose groups. For example, average ductal area was 45% larger in the NOAEL treated group compared to controls.
Whole mount adult female mammary glands were evaluated for the volume fraction of ducts, terminal ends, alveolar buds, and the sum of all epithelia (Figure 4). The average volume fraction (VF) of ducts, terminal ends, alveolar buds, and all epithelium for the control group was 0.31, 0.05, 0.001 and 0.36, respectively. The 95th

2.4.4 Adult Female Whole Mount Analysis

Whole mount adult female mammary glands were evaluated for the volume fraction of ducts, terminal ends, alveolar buds, and the sum of all epithelia (Figure 4).

Figure 2.3. Pubertal female whole mount analysis results of ductal area (sq mm), ductal extension (mm), number of TEBs, total TEB area (sq mm), average TEB size, and TEB density. Data is considered significant if the ANOVA p-value < .05. Control N=7, 95th Percentile N=7, TDI N=10, NOAEL N=10. (A) Ductal extension was not significantly different between treatment groups (p=.124). (B) Ductal area was not significantly different between treatment groups (p = .117). (C) The number of TEBs was not significantly different between treatment groups (p = .713). (D) Total TEB area was not significantly different between treatment groups (p = .641). (E) Average TEB size was not significantly different between treatment groups (p = .154). (F) TEB density was not significantly different between treatment groups (p = .396).
percentile exposure group also had a sample size of 9. The average VF of ducts, terminal ends, alveolar buds, and all epithelium was 0.30, 0.04, 0.01, and 0.35, respectively. The TDI group had a sample size of 10. The average VF of ducts, terminal ends, alveolar buds, and all epithelium was 0.33, 0.05, 0.01, and 0.38, respectively. Lastly, the NOAEL group had a sample size of 10. The average VF of ducts, terminal ends, alveolar buds, and all epithelium was 0.29, 0.03, 0.01, and 0.32, respectively.

After analysis using ANOVA, there were no significant differences in VF ducts (p = 0.526) or VF all epithelium (p = 0.301). While not statistically significant, the VF alveolar buds were shown to increase in a monotonic dose-dependent manner (ANOVA, p = 0.137). The only significant difference between groups was for the measure of VF terminal ends (p = 0.020). Post hoc analysis identified a statistically significant difference between the NOAEL group and the control (p* = 0.010), 95th percentile (p* = 0.019) and TDI (p* = 0.007) groups, respectively.
Figure 2.4. Adult Female Whole Mount analysis results of volume fraction (VF) ducts, VF terminal ends, VF alveolar buds, VF all epithelia.

Treatment groups are control, 3 μg/kg (95th percentile), 500 μg/kg (Tolerable Daily Intake [TDI]), & 18000 μg/kg (NOAEL).

(Control N=9, 95th Percentile N=9, TDI N=11, NOAEL N=11)

(A) VF ducts were not significantly different between treatment groups (p = .777). (B) ANOVA revealed significant differences in VF terminal ends between treatment groups (p = .027). Post hoc analysis showed that the NOAEL treatment group is the only group that significantly differs from all other treatment groups: control (p* = .01), 95th percentile (p* = .019), & TDI (p* = .007). Post hoc tests showed no differences between control, 95th percentile, and TDI groups. (C) VF alveolar buds was not significantly different between treatment groups (p = .098) (D) VF all epithelium was not significantly different between treatment groups (p = .562) P* < 0.05, fisher post hoc test
2.4.5 Immunohistochemistry of Adult Female Mammary Gland

Sectioned adult female mammary glands were evaluated for their expression of Ki67, ER, and PR (Figure 5). Raw data were used to calculate the percentage of cells expressing the marker of interest (% positive). Analysis of Ki67, a marker of proliferation, revealed no significant differences between treatment groups (ANOVA, p = 0.602), although we noted an increase in the expression of this marker with increasing dose of BBP (Figure 5A). There was also no significant effect of BBP treatment on the percentage of epithelial cells expressing ERα (ANOVA, p = 0.778; Figure 5B). In contrast, there was a significant effect of treatment on the percentage of epithelial cells that were positive for PR (ANOVA, p = 0.025; Figure 5C). Post hoc analysis revealed that there was significantly more PR expression in the TDI group compared to the 95th percentile of exposure (p* = 0.010) and NOAEL groups (p* = 0.008). However, the 95th percentile group and NOAEL group did not significantly differ from each other (p* = 0.996).
Figure 2.5. **Percent positive cells expressing each immunohistochemistry marker of interest (Ki67, Estrogen Receptor, & Progesterone Receptor) across treatment groups.** (A) The percent of cells expressing Ki67 was not significantly different between treatment groups (p = .602) (Control N=9, 95th Percentile N=9, TDI N=11, NOAEL N=9). (B) The percent of cells expressing ER was not significantly different between treatment groups (p = .778) (Control N=9, 95th Percentile N=9, TDI N=10, NOAEL N=10), (C) The percent of cells expressing PR was significantly different between treatment groups (p = .025) (Control N=9, 95th Percentile N=9, TDI N=10, NOAEL N=10). Post hoc analysis revealed a statistically significant difference between the TDI and NOAEL groups (P** = .008). There is also a significant difference between TDI and 95th percentile groups (P* = .01) P* < 0.05, fishers post hoc test.
Figure 2.6 Quantification of proliferation index in mammary epithelial cells. Images taken at 400x magnification illustrating expression of Ki67. Purple cells are negative for Ki67, counterstained with hematoxylin.
Figure 2.7 **Quantification of Estrogen Receptorα expression.** Images taken at 400x magnification illustrating expression of ERα. Purple cells are negative for Erα counterstained with hematoxylin.
2.5 Discussion

At the time of necropsy, we collected body weight and anogenital distance, and then calculated anogenital index (which accounts for differences in body weight) for both males and females at puberty and in adulthood. We were surprised to find significant differences in female body weight that persisted from puberty (ANOVA, p=0.002) to

Figure 2.8 Quantification of progesterone receptor expression mammary epithelial cells. Images taken at 400x magnification illustrating expression of PR (brown cells, brown arrow). Purple cells (noted by blue arrow) are negative for PR, counterstained with hematoxylin
adulthood (ANOVA, p < 0.001). Post Hoc analysis for multiple comparisons showed that the pubertal weight significance was largely driven by the NOAEL treatment group compared to the control. Furthermore, post hoc analysis revealed statistical significance in body weight difference when comparing the NOAEL group to the 95th percentile of exposure (p* = 0.003), and TDI (p* = 0.004) groups, respectively. The significant differences in body weight measured in adult females were similar to those observed at puberty, although the effects were more distinct. All post hoc comparisons were significant with the exception of the comparison between the 95th percentile of exposure group and TDI group (p* = 0.653). Collectively, these results suggest a strong dose-dependent effect of BBP on body weight, as well as sex-specific effects on this outcome (with females significantly affected whereas males were not). A summary of significant findings can be found in the table below (2.1).

2.1 Summary of significant findings in evaluation of BBP on pubertal female, adult female, pubertal male, and adult male mammary glands

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<th>Ductal Ext.</th>
<th># TEB</th>
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<th>Avg TEB size</th>
<th>TEB Density</th>
<th>VF Ducts</th>
<th>VF TE</th>
<th>VF AB</th>
<th>VF Total Epithel.</th>
<th>% Pos Kc67</th>
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No significance
Trending toward significance (ANOVA, 0.15 < p < 0.05)
Trending toward significance with post hoc p < 0.1 compared to control (a), 95th percentile (b), TDI (c), and NOAEL (d)
Significant in ANOVA (p < 0.05)
Significance in ANOVA and Fisher post hoc (p* < 0.05) compared to control (a), 95th percentile (b), TDI (c), and NOAEL (d)
Not evaluated
Alterations to female body weight following early life exposure have not been previously reported in *in vivo* studies of BBP. The Russo group evaluated gestational BBP exposure and noted no changes to body weight. However, a 3-day uterotrophic assay conducted in adult mice noted that BBP exposure significantly increased body weight at the lower dose (20 mg/kg) and although the same trend was noted in the high dose group (200 mg/kg) the difference was not found to be significant (Ahmnad et al. 2015). When these authors conducted a 20-day pubertal female assay, they instead noted a significant decline in body weight at both dose levels throughout the study period.

Increased body weight is an outcome relevant to obesogen exposures. The current obesogen hypothesis is that they are environmental chemicals the promote obesity via increasing adipocyte commitment, differentiation or size. This occurrence can be largely attributed to altered metabolic setpoints or hormonal regulation (Egusquiza & Blumberg, 2020). While obesogens are now recognized as a specific hazard for environmental chemicals, many obesogens are also EDCs which have obesogenic properties via alteration to hormonal pathways (Egusquiza & Blumberg, 2020, Heindel & Blumberg, 2019). The endocrine system plays major roles in metabolism, fat deposition and distribution. Specifically, estradiol disruption can contribute to increased body weights via effects on food intake, lipogenesis, fat distribution, or alterations to the balance of glucose and insulin (Heindel & Blumberg, 2019). Thus, body weight may not be a sole indicator of endocrine disruption, but it may contribute to evidence of endocrine disrupting effects.

Previous studies on EDCs have demonstrated that exposures can increase body weight, consistent with obesogenic effects as well as alterations to metabolic pathways.
involving endocrine signaling (e.g., PPARy regulation of lipid metabolism) (Heindel et al., 2015). One epidemiological study measured phthalate exposures by quantifying the urinary metabolite MBzP and found a positive association between urine MBzP concentration and BMI, specifically in female children aged 8-15 (Katsikntami et al. 2016). Conversely, a cohort study in China found that there was no association between MBzP monoesters in amniotic fluid and the weight, length, gestational age, or AGI of newborn females (Huang et al., 2009). The genomic analysis conducted by the Russo group proposes an interesting mechanism behind the current finding of alteration to body weight. Moral et al. highlighted the possibility that BBP could act as a metabolism disrupting compound as it was shown to dysregulate several metabolic pathways. Particular attention is drawn to the increasing evidence of EDCs interacting with the PPAR family, an active participant in lipid and carbohydrate metabolism. Metabolic pathways are highly complex and susceptible to dysregulation, like known hormonal pathways.

In contrast to the effect of BBP on body weight, there were no significant effects of BBP on anogenital index in either males or females. Anogenital index is a reproducible measure that is influenced by prenatal androgen exposures. Endogenous androgens cause a lengthening of the anogenital distance in males, and anti-androgenic chemicals including several phthalates have been shown to decrease anogenital index in male rodents exposed during prenatal development. Our results suggest that BBP is not acting as an anti-androgen in these animals. Or alternatively, BBP is not sufficiently potent to alter this reproductive outcome.
In depth evaluation of the female mammary gland developmentally exposed to BBP yielded both significant and null findings that will further contribute to the growing breadth of evidence for BBP acting as an EDC. Whole mount analysis of female pubertal mammary glands yielded no significant effects of perinatal BBP exposures, although ductal area and ductal extension increased with increasing dose of BBP. Further investigation with larger sample sizes is needed to determine if this observation is random or treatment related. However, the larger mammary glands observed in BBP-exposed animals are similar to the outcomes that have been observed in mice following perinatal exposures to some estrogenic EDCs like bisphenol A (Markey et al., 2001) and bisphenol S (Kolla et al., 2018).

Adult whole mount analysis revealed a statistically significant difference in the number of terminal ends (also referred to as terminal ducts). The number of terminal ends observed in the NOAEL group significantly varied from all other treatment groups. Interestingly, the number of terminal ends decreased in this treatment group, suggesting that there was a decrease in lateral branching in females that had been developmentally exposed to the highest dose of BBP. This outcome may be indicative of two effects. Decreased number of terminal ducts could occur due to reductions earlier in development (e.g., fewer TEBs at puberty), although our results do not support this possibility. Alternatively, there may be fewer terminal ducts due to reduced secondary branching. Qualitative observations of the whole mount mammary glands do suggest that there was a decrease in lateral branching in females exposed to the highest dose of BBP, although a quantitative analysis of this outcome is needed to verify our observations. Additional studies are also needed to quantify expression of genes involved in epithelial branching.
While not statistically significant, the volume fraction of alveolar buds appeared to be increasing with increasing dose of BBP. Again, further investigation with a greater sample size is necessary to determine if this is a random or important trend. Importantly, increases in the number of alveolar buds have been reported in other studies of estrogenic EDCs including studies of bisphenol A, bisphenol S, and oxybenzone (Vandenberg et al, 2008, Kolla et al 2018, Matouskova et al, 2020).

We used immunohistochemical analyses to examine two hormone receptors, ERα and PR, in the mammary epithelium. This approach revealed significant findings in expression of PR. Specifically, the TDI group had significantly greater PR expression compared to the 95th percentile and NOAEL groups, although PR expression did not significantly vary between control and exposure groups. Findings on altered PR expression in the mammary gland are interesting because progesterone promotes the development of lobular-alveolar structures and functions necessary for milk secretion (Teravborelli, 2015), and as discussed above, we saw a non-statistically significant effect of BBP on VF alveolar buds. Previous research on BBP evaluated the effects of this chemical on PR-mediated signaling. One study using MCF-7 cells demonstrated a concentration dependent increase in PR following BBP administration (Picard et al. 2001). Another study evaluated the effects of BBP on in adult female rat and found a significant increase in PR expression mRNA within the preoptic area (Funabashi et al., 2001). Observed changes in PR expression may be explained by direct and indirect effects on the receptor: increased expression of PR may be observed in response to an increase in progesterone hormone in the blood stream or it may be caused by factors initiating the transcription and/or translation of the gene responsible for PR expression.
PR has several isoforms and can additionally function in a ligand-dependent and ligand-independent manner. In the ligand dependent pathway, progesterone binds to the PR resulting in a conformational change that activates the receptors’ function (Azeez, 2021). This complex dimerizes and binds to progesterone response elements in target genes. The bound complex then recruits transcription factors and coactivators, thus regulating expression of the target gene. The observed increase in PR when mice were exposed at the TDI dose could provide more opportunity for ligand binding and increased activation of gene expression. Future studies should explore this possibility. Progesterone and PR signaling acts on RANKL, a paracrine mediator (Obr & Edwards, 2012). While altered PR expression alone observed in this study cannot be conclusively deemed an adverse effect, it is important to note that increasing attention is being drawn to altered progesterone signaling and breast cancer association. A breast cancer phenotype includes PR positive tumors, which express PR and are susceptible to progesterone signaling as it may accelerate development (Brisken, 2013). PR can also operate in a ligand-independent pathway that is important for signaling cascades and receptor crosstalk (Azeez et al., 2021). The observed increase in PR among TDI exposed females and the known ligand-independent pathway of PR leaves room for signal dysregulation even in the absence of excess circulating progesterone. Additional studies are needed to distinguish these possibilities.

We observed an increase in the percentage of epithelial cells expressing the proliferation marker Ki67, which appears to increase with dose of BBP, although this finding was not statistically significant. These results conflict with the decreased number of terminal ends observed in whole-mount mammary glands. To further understand the
effect of BBP on proliferation, future studies will need to quantify proliferation at earlier ages (including puberty).

The only other laboratory to study the effects of gestational BBP exposure on the mammary gland is the Russo group (Moral et al. 2011). The purpose of their study was to clarify the effect of prenatal exposure to BBP on the morphology, proliferative index, and genomic signature of the mammary gland. That study utilized the rat model which has been largely established in toxicology and risk assessment as a model for human metabolism. Compared to the mouse model, the rat has a more complex mammary structure that is structurally more reflective of human mammary gland development however varies in terms of hormonal pathways and regulation of development. Rats were treated with either 120 mg BBP/kg body weight or 500 mg BBP/kg body weight. These doses are much higher than those used in the current study in which mice were dosed with BBP via oral feeding in corn oil at 0.003 mg/kg, 0.5 mg/kg or 18 mg/kg. The approaches that were used to evaluate the mammary gland were similar in both our study and the Russo study. In the rat study, pups were evaluated at four different time points: PND 21, 35, 50 and 100; these time points span from pre-puberty to early adulthood. In our study, mammary glands were evaluated only at puberty (n=7-10) and adulthood (N=7-10).

In the rat model, when compared to the control, researchers found that prenatal exposure to the high dose of BBP increased the number of terminal ducts observed at PND 21. In contrast, the present mouse study found decreased terminal ducts in the NOAEL dose group (ANOVA, p=0.02) and these effects were noted in adult samples. Additionally, the rat study reported an increase in alveolar buds compared to the control.
at PND 35. While not statistically significant, the adult mouse whole mount analysis also indicated a trend toward increased volume fraction alveolar buds. The final finding noted in the rat whole mounts was in TEBs. While not statistically significant, there was an increase in TEBs at day 50 among the high dose group (p= 0.093). Interestingly, gross observation of adult mouse mammary glands revealed some remaining TEB-like structures in the adult mammary gland. Future studies are needed to definitely determine if these structures are truly TEBs, or if they are intraductal hyperplasias, as has been observed in studies of complex EDC mixtures (Sapouckey et al., 2018). Future studies are also needed to determine if these BBP-exposed females have enhanced responses to other carcinogenic challenges, which would be an important adverse effect to evaluate.

In the rat model, alterations to proliferation were observed in a structure dependent manner (e.g., in ducts, terminal ends, alveolar buds, and Lob1 structures). In the current mouse study, all epithelial structures were evaluated for proliferation together. The only significant findings associated with BBP exposure to the rat model relevant to proliferation was at PND 100, where the low-dose BBP group had a higher percent proliferating cells in ducts and terminal ducts compared to the control group. Additionally, at PND 100, the high-dose BBP group was found to have greater percent proliferation in Lob1 structures compared to the control group. Based on their findings, the Russo lab group concluded that BBP significantly affects the postnatal maturation of the female rat mammary gland. Their findings were dose and age-dependent indicating a vast potential for dysregulation caused by BBP exposure.
Delayed vaginal opening and other gross indicators of endocrine disruption or delayed puberty were also reported in this rat study. Unfortunately, our study on BBP and the mouse mammary gland did not record data on the day of vaginal opening.

2.6 Conclusion

Our experiment evaluating the effects of gestational exposure to BBP yielded minimal findings in pubertal mice but significant findings in adult female mice consistent with endocrine disruption. BBP belongs to a class of compounds (phthalates) that have gained recent attention for their endocrine disrupting. Phthalates such as BBP are plasticizers used ubiquitously throughout the modern environment. BBP is commonly found in PVC piping, vinyl flooring, and carpet backing (among other plastic products). There is limited existing evidence of the effects BBP may have on mammals and only one lab group had previously evaluated the effects of BBP on the mammary gland (Moral et al., 2007 & 2011). Evaluation of the mammary gland is particularly relevant in the evaluation of BBP as there are previous findings of effects on this organ, including disruptions to the rat mammary gland morphology.

In this thesis, mice were orally administered one of three doses of BBP via corn oil. Control animals were solely administered the oil vehicle. Mice were dosed daily throughout pregnancy and lactation. Pups were fed a normal diet (without BBP) after weaning. A portion of pups were raised to puberty before sacrifice for pubertal analysis. The remainder were raised until young adulthood and then sacrificed for analysis. All animals were weighed, and the 4th inguinal mammary gland was collected upon sacrifice. Mammary glands were evaluated using both whole mount and immunohistochemical analysis.
As hypothesized, utilization of the mammary gland in analysis of BBP yielded further evidence of endocrine disrupting potential, particularly in progesterone receptor expression and other growth parameters dependent on hormone signaling. We observed significant changes increases in female body weight among females in the highest treatment group that persisted from puberty to adulthood. In the adult mammary gland, we observed a significant decreased terminal duct in the highest treatment group. Additionally, there was a significant alteration to progesterone receptor expression. The effects on PR expression are similar to the non-monotonic dose response curves commonly observed in studies of EDC chemicals, although the mechanism for this effect in our study has not yet been elucidated.

While findings and alterations to hormonal receptor expression contribute to evidence of endocrine disruption with BBP exposure, these findings are not conclusive on their own. Ultimately, there are limitations to this study that could have affected the results. Primarily, sample sizes of analysis were low (n= 7-10). Increasing the sample size should reduce variability and increase statistical power. Further research should also aim to verify findings from other studies. However, due to the inconsistency in existing research, precaution should be taken to ensure that future experiments include adequate samples for analysis. Furthermore, researchers should investigate endpoints that are relevant to progesterone receptor mediated signaling and terminal duct development.
CHAPTER 3

OECD EXTENDED ONE-GENERATION REPRODUCTIVE TOXICITY GUIDELINES

3.1 Introduction

The Organization for Economic Co-operation and Development (OECD) is an intergovernmental collaborative organization comprised of 34 countries across the globe, all committed to coordination and harmonization of research and policy of mutual concern. The OECD fosters collaboration on science, economics, and policy by providing guidance on relevant topics and methods for research (OECD GD 151, 2013). Three major topics of interest to the organization include reproductive toxicity, developmental toxicity, and endocrine disruption. The OECD has developed various test guidelines which provide universal methods of analysis for different endocrine-related systems/outcomes of interest (e.g., OECD TG 422 for reproductive toxicity; TG 440 which describes the Uterotrophic assay, a measurement tool to identify estrogenic chemicals; TG 441, which describes the Hershberger assay, a measurement tool to identify anti-androgenic chemicals). The Extended One Generation Reproductive Toxicity (EOGRT) Guideline (TG 443) was developed by the OECD based on established regulatory tests from the International Life Science Institute-Health and Environmental Institute, and the Agricultural Chemical Safety Assessment Committee. The EOGRT study design improves and clarifies the existing generational studies in a study design that provides flexibility to the researcher. Trigger points are used in the EOGRT design to be more conservative with materials, animals, and time. For example,
observation of altered behavior in pups may trigger further evaluation into brain morphology. Alternatively, if researchers do not observe any gross reproductive abnormalities, hormonal alterations, or behavioral changes, they would not proceed to evaluate the mating and reproductive abilities of the F1 offspring. The EOGRT study also urges researchers to draw on previous literature to determine dosing or endpoint that should be included or omitted. The main research objective of the EOGRT study is to evaluate exposures and life stages that may not have been covered in other toxicity tests: pre- and postnatal exposure, toxicity in pregnant and lactating females (rodent), pubertal, adult, and reproductive (if triggered) outcomes in offspring (OECD TG 443, 2018).

### 3.1.2 The importance of the mammary gland in evaluating reproductive toxicity, developmental toxicity, and endocrine disruption.

The mammary gland is important to include in reproductive and developmental toxicity for a multitude of reasons. The most prominent reason is that while considered to be a secondary reproductive organ, a normally structured and functioning mammary gland is essential to the sustenance of newborns. Secondly, the hormone-dependent development of the mammary gland can provide valuable information to researchers on hormonal alterations during key windows of development such as gestation, puberty, and pregnancy. Finally, there is increasing evidence of developmental exposures increasing one’s susceptibility to cancers later in life (Manibusan & Touart, 2017). Currently in the OECD EOGRT guidelines the mammary gland is considered a secondary organ and the guidance only recommends collection and evaluation of the organ if previous research indicates the likelihood of effects on the organ. The guidelines do not specify which existing findings would trigger mammary gland evaluation, rather, it is left to the
interpretation of the researcher. The methods for mammary gland evaluation are not outlined in the published guideline study. However, guidance is published in the OECD guidance documents (OECD GD 151, 2013) which may be utilized alongside the general test guidelines. The mammary gland evaluation methods outlined by the OECD will be discussed later in this chapter.

3.2 The EOGRT study

The TG outlines in detail each element of the study including animal species and strain selection, housing, feeding conditions, route of administration, dosing, and endpoints to be evaluated in relation to reproduction and development. In brief, the guideline outlines a preference for a dietary route of exposure and recommends an aqueous solution as the vehicle for the compound of interest. To comply with this TG, a study should include at least three dose levels and one (negative) control group. Doses should be selected based off existing toxicokinetic data, and if these are not available dose levels should be selected based on toxic effects observed during a range-finding study. Dosing should begin in the parental generation at least two weeks prior to mating and should continue daily through the mating period. Exposures should continue in females of the parental generation through pregnancy and lactation and males of the parental generation should be administered the test compound for a similar length of time (approximately 10 weeks total), even after they are removed from the cages with the females. Upon weaning, the F1 generation offspring will be directly exposed to the test compound until necropsy in adulthood. For any F1 offspring that are used to evaluate fertility outcomes, exposures will continue until weaning of the F2 generation.
Within this TG, there are numerous endpoints for evaluation that have been separated into 5 main categories: clinical biochemistry, sperm parameters, gross organ morphology, organ weight, and histopathology of target organs. These outcomes are described in more detail below. However, the OECD emphasizes throughout the TGs that elements of the study design or analysis can be modified at the discretion of the experimenter.

During mating and pregnancy, researchers should record the dates of pairing, date of insemination, the date of parturition, and then calculate the duration of pregnancy. Upon the birth of each litter, specific parameters should be recorded including the number and sex of pups as well as the number of still and live births. Pups should be examined for the presence of gross abnormalities as well as an assessment of body temperature, activity level, and reaction to handling. Between PND 0 and 4, anogenital distance (AGD) should be recorded followed by recording of the presence/absence of nipples/areolae in male pups on PND 12 or 13. Urine samples should be taken from all animals before necropsy to evaluate the appearance, volume, osmolality/specific gravity, pH, protein, glucose, blood and blood cells, cell debris, and known metabolites of the compound of interest.

TG 443 lists a number of analytical and observational components that should also be included for all animals, regardless of cohort. Firstly, there are several in-life observations to be recorded by the investigator. Clinical observations should be noted in both the parental and F1 generations including any morbidity/mortality, behavioral changes (e.g., self-mutilation, seizures, walking backwards, stereotypies, the presence of clonic or tonic movements, etc.), or notable changes to external appearance that may be
indicative of toxic effects such as abnormal fur color, barbering, or the presence of skin rashes. Additional in-life observations include regular body weight records and food and water consumption. Finally, the guidelines recommend observing the estrous cycle via vaginal cytology of dams and female offspring.

TG 443 outlines that the F1 animals be split into 6 cohorts of animals that are designated for evaluating different outcomes: Cohorts 1a and 1b are designated for reproductive evaluation, 2a and 2b are designated for neurotoxicity outcomes, 3 is designated for immunotoxicity evaluation, and lastly there is a surplus cohort for spare or additional sampling. Sample sizes for each of these cohorts are 10 or 20 male and female pups from the F1 generation, depending on the cohort being evaluated. Further description of the EOGRT study cohorts is provided below.

Figure 3.1 Extended One-Generation Reproductive Toxicity (EOGRT TG 443) Schematics. Summary of F0 exposure from pre-mating through lactation, F1 cohort selection, and age at necropsy.

3.2.1 Parental evaluation

Females of the parental generation should have their estrous cycles observed from the beginning of treatment via vaginal smears. Additionally, they should be observed for
standard endpoints as well as variables relevant to mating and pregnancy. Such endpoints of interest include dates of pairing, date of insemination, date of parturition, precoital interval, and duration of pregnancy. Additionally, researchers should note any signs of dystocia or abnormal maternal nesting or nursing behaviors. Terminal observations are to be conducted in 10 randomly selected males and females from each treatment group. These mice should be evaluated with full or partial hematology, clinical biochemistry, serum T4 and TSH assays and other examinations consistent with current literature. Hematological parameters should include hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte counts, platelet count, and blood clotting time/potential. Serum investigation should include glucose, total cholesterol, urea, creatinine, total protein, albumin, and two enzymes indicative of kidney function. Sperm parameters of the parental generation should also be assessed via collection, weight, and histopathology of the testis and epididymis. Additionally, the epididymis should be evaluated for sperm motility and morphologic abnormalities. Histopathological analysis should be conducted on tissues from the F0 generation. Primary organs including uterus, ovaries, testis, epididymites, prostate, seminal vesicles, brain, liver, kidneys, heart, spleen, thymus, pituitary, thyroid, and adrenal glands should be collected. The guidelines additionally note a secondary list of organs to be collected and preserved “under appropriate conditions.” These tissues are peripheral nerve, muscle, spinal cord, eye plus optic nerve, gastrointestinal tract, urinary bladder, lung, trachea, bone marrow, vas deferens (male), mammary gland (males and females), and vagina (female).
3.2.2 Cohort 1A

One male and one female per litter per group are selected for this cohort (20/sex/group). These pups are evaluated for primary assessment of effects on reproductive systems and general toxicity. Vaginal smears should be examined daily once vaginal patency (opening of the vaginal cavity) is observed. Following the initial observation of estrous cycle, female pups in this cohort should be evaluated for regular estrous cyclicity for two weeks beginning around PND 75. Organs should be collected and preserved at the discretion of the researcher for histopathology although full histopathology should be conducted on all control and high-dose animals from cohort 1A. Organs to be evaluated are uterus, ovaries, testis, epididymites, prostate, seminal vesicles, brain, liver, kidneys, heart, spleen, thymus, pituitary, thyroid, and adrenal glands. Similar to the parental generation, the peripheral nerve, muscle, spinal cord, eye plus optic nerve, gastrointestinal tract, urinary bladder, lung, trachea, bone marrow, vas deferens (male), mammary gland (males and females), and vagina (female) may be collected if the current study or previous research indicates there may be effects. Following the examination of organs in the lower dose groups, researchers should conduct histopathology on tissues demonstrating treatment-related changes.

Additionally, the 1A cohort should be investigated both pre- and postnatally for induced immunotoxic effects. Immunotoxicity analysis begins with weighing the lymph nodes that are associated with the route of exposure. Additionally, researchers should collect lymph nodes that are distant from the route of exposure. Using one-half of the preserved spleen, the guidelines outline splenic lymphocyte subpopulation analysis. Lymphocytes of interest are CD4+, CD8+ T lymphocytes, B lymphocytes, and natural
killer cells (NK). The other half of the spleen, lymphoid organs, bone marrow, thymus, and adrenals should undergo histopathological analysis for further evaluation of immunotoxic effects.

3.2.3 Cohort 1B

One male and one female per litter per dose group (20/sex/group) are selected to provide follow-up assessment of reproductive performance, obtaining additional data if endocrine disrupting effects are indicated, or when results from cohort 1A are equivocal. While all organs listed in cohort 1A should be collected and preserved, reproductive and endocrine related tissues are specified for processing and analysis in cohort 1B. The organs to be evaluated from this cohort include the vagina, uterus with cervix, ovaries, testes, epididymites, seminal vesicles and coagulating glands, prostate, pituitary, and other identified target organs. Ovary evaluation should aim to quantify primordial and small growing follicles and the corpora lutea. Assessment of these organs should be informed by previously mentioned estrous cyclicity testing as there can be slight differences in tissue appearance depending on where the animal is in its estrous cycle. Oviducts, uterus, and vagina should be examined for any abnormal development. Testicular histopathology should be conducted to identify alterations to testis differentiation or development. The corpus, epididymis, and vas deferens should be observed for abnormal development.

3.2.4 Cohort 2A

20 pups per group (1 male and 1 female from each litter) are selected for neurobehavioral testing in adulthood (PND75-90) in cohort 2A. Cohort 2A is subjected to neurological tests such as auditory startle, functional observational battery, motor
activity, and a neuropathology assessment. Following termination, the brain should be weighed and subjected to microscopic examination for neurotoxicity assessment. Histopathological analysis of the brain tissue should be conducted on high dose and control groups unless treatment related differences are observed in gross evaluation of the tissue. The brain should be sectioned such that there can be analysis of the olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, mid-brain (thecum, tegmentum, and cerebral peduncles), brain stem and spinal cord. In studies of the animals allotted to cohort 2A, researchers should additionally investigate the eyes (retina and optic nerve), peripheral nerve, muscle, and spinal cord. Evaluation of these tissues should be conducted per OECD TG 426

3.2.5 Cohort 2B

20 pups per group (1 male and 1 female from each litter) are designated for neurohistopathology assessment upon weaning at PND 21 or 22. Following euthanasia, the brain should be weighed and subjected to microscopic examination for neurotoxicity assessment. Histopathological analysis should be conducted on the high-dose and control animals unless gross anomalies are observed upon primary analysis. The brain should be sectioned such that there can be analysis of the olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, mid-brain (thecum, tegmentum, and cerebral peduncles), brain stem and spinal cord. Evaluation of these specified tissues should be conducted per OECD TG 426.
3.2.6 Cohort 3

20 pups per group (1 male and 1 female from each litter) are selected for cohort 3 if sufficient numbers of pups are available. These pups are euthanized after weaning unless previous cohort analysis indicates a need for further in-life observations. At a minimum, the brain, spleen, and thymus should be collected and weighed from cohort 3 animals. Gross anomalies should be noted, and target tissues should be preserved. Collection of mammary tissues from this cohort for further analysis is also recommended. These additional pups can be used for other experimentation specifically in the T-cell dependent antibody response assay.

3.3 Purpose

This chapter aims to identify and summarize the existing published studies that utilized OECD TG 443. We determined which of the outcomes in TG 443 were included and which of the outcomes were significantly affected by exposures to test chemicals. Following evaluation of these studies, we next aimed to determine whether mammary gland outcomes were examined in any published study. Critics claim that adding the mammary gland to the EOGRT study is not feasible as there are no standard validated methods of analysis, or by claiming that evaluation of the mammary gland would not improve or contribute to the EOGRT study, or that inclusion of this organ would unreasonably increase the time and cost of the study. Completion of this work will assist mammary gland researchers in addressing those claims raised by those opposed to improved mammary gland parameter inclusion in TG 443.
3.4 Methods

3.4.1 Evaluation of OECD TG 443 (Extended One-Generation Reproductive Toxicity)

The document outlining the Extended One-Generation Reproductive Toxicity (EOGRT) study design in addition to the guidance (OECD GD 151, 2013) and supporting document (OECD GD 43, 2013) was reviewed. Endpoints evaluated in the EOGRT study were organized into a table detailing each endpoint. We also create a summary time with the estimated amount of time each test may take before or after necroscopy (Table 3.1).

3.4.2 Study collection

A comprehensive summary of TG 443 was followed by the systematic evaluation of current published studies developed using the guidelines from 2011 to present. To ensure collection of all studies utilizing OECD TG 443, multiple databases were searched. Studies were sourced from the National Toxicology Program, NCBI, UMass library database, google scholar, and regulations.gov. Each database was searched for various terms such as “extended one-generation”, “One-generation reproductive toxicity”, “EOGRT”, “TG 443”, “OECD TG 443.” Results were then cross-referenced and organized into a table.

3.4.3 Study Evaluation

Studies were first reviewed for their methods of analysis. The compound of interest, doses utilized, endpoints of interest, and notable findings were collected.
Measured endpoints evaluated by each study were compared to those required by the TG in a comprehensive table with the aim of cross tabulating which endpoints outlined in the OECD test guidelines were utilized by researchers conducting the studies (Table 3.2). Finally, for each endpoint, another table was created to illustrate studies that observed significant outcomes related to the endpoints (Table 3.3). To further evaluate the EOGRT studies and study design for EDC investigation, a table was created listing all outcomes from TG 443 that may indicate endocrine disruption and if the endpoint was evaluated and/or an outcome observed (Table 3.4).

3.5 Results

3.5.1 Summary of published studies using TG 443

9 published studies cited the use of OECD TG 443 Extended One-Generation Reproductive Toxicity guidance, and another 2 unpublished studies were referenced in what was referred to as a feasibility study (Fegert et al., 2012). As a first step, we summarize each of these studies including any deviations that were made from the test guideline, and any significant results that were obtained.

3.5.1.1 Benzoic Acid (Turnbull et al., 2021)

Benzoic acid (BA) is a common preservative used in processed foods because it inhibits bacterial, mold and yeast growth. In the first study, the effects of benzoic acid were evaluated using methods that closely adhered to the EOGRT study. Previous generational studies found an acceptable daily intake for BA of 5 mg BA/kg bw/day (Kieckebusch & Lang 1960). BA was examined using the EOGRT design to understand a broader range of BA exposures. Animals were administered BA via incorporation of BA into the dry food at 0, 7500, 11500, and 1500 ppm. However, although the study reports
that it evaluated hematology outcomes, included serum analysis, and measured organ weights as per the guidelines, the published study does not specifically note the endpoints evaluated.

The lack of detail provided may have influenced interpretation of the outcomes that were reported for BA. Considering this limitation, the study reported no significant findings in either the F0 or F1 generations.

3.5.1.2 Ni-n-octyltin dichloride (DOTC) (Tonk et al., 2011)

DOTC is used as an intermediate in the production of organotin compounds; importantly, several organotins have endocrine disrupting properties (Kirchner et al., 2010, Li et al., 2011, Chamoro-Garcia et al., 2017). DOTC is also used as an herbicide to control grass weeds. The EOGRTs study evaluating DOTC was incomplete as this study was used as a validation study for the developmental immunotoxicity component of TG 443. The DOTC study therefore included the collection and weight of the testes, liver, kidneys, heart, spleen, thymus, pituitary, and adrenals in males only. For immune assessment, blood samples were taken from the femoral shaft and the spleen and thymus were evaluated for their cellular composition as per their description in the TG. In brief, researchers evaluated the lymphocyte subpopulation, splenocyte population, splenic lymphoproliferative response and splenic nitric oxide production Both primary and secondary IgM and IgG response to KLH were measured to evaluate the delayed-type hypersensitivity (DTH) response.

Several significant effects were observed following exposures to DOTC. First, increased body weight was measured in DOTC-exposed dams during the lactation period. Additionally exposed dams showed increased post-implantation pregnancy loss based on
a comparison of the number of live births and the number of uterine implantation sites. In the F1 generation, significant effects observed in the high dose group included increased mortality rates and increased body weight in early development (i.e., at postnatal day PND 8 -13). At necropsy conducted at PND21 or PND42, F1 males exposed to the highest dose had decreased thymus weight with decreased cellularity, and the blood hematocrit revealed a decreased platelet volume in males. F1 males from the mid and low dose groups, but not the highest dose, showed increased liver weights. Compared to the age-matched controls, males from all DOTC treatment groups showed altered splenocyte subpopulations as well as altered cytokine production. Finally, DOTC-treatment groups demonstrated an increased DTH response to the immunotoxicity KLH test, an indication of immune hypersensitivity.

3.5.1.3 2,4-dichlorophenoxyacetic acid (2,4-D) (Marty et al., 2013)

Historically, 2,4-D was an herbicide widely used for the control of broadleaf weeds. Agent Orange, an infamous defoliant used during the Vietnam War, contained both 2,4-D and another herbicide, 2,4,5-T. Agent Orange was banned from use due to the unintended contamination of the mixture with dioxin, a byproduct of 2,4,5-T. This study exposed rats to 0, 100, 300, 600 (female) or 800 (male) ppm 2,4-D in their feed. While the EOGRT study evaluating 2,4-D evaluated all primary organs outlined in the guideline, there were still important elements of the study missing such as the absence of detail of either hematological or serum analysis conducted.

F0 female dams from the high dose 2,4-D group had decreased body weights during the lactational period and F1 pups also had decreased weights throughout the lactation period. Researchers found signs of kidney toxicity including increased kidney
weights in female exposed groups at the highest dosage, renal lesions, as well as changes in kidney histopathology, although the differences between treatment groups and controls were not statistically significant. The main significant findings were observed in the reproductive tracts of F1 males including decreased seminal vesicle and prostate weights among 2,4-D treatment groups as well as a slight delay in preputial separation.

3.5.1.4 Ethanol (Tonk et al., 2013)

While ethanol (EtOH) has been shown to cause fetal toxicity in numerous animal models and epidemiological studies, the EOGRT study performed with EtOH was focused on validating the sensitivity of the immune and developmental parameters of the EOGRT study design. Adult Wistar rats were exposed to EtOH via their drinking water at 0, 1.5, 4, 6.5, 9, 11.5 and 14% w/v. Unfortunately, there were high rates of toxicity and mortality observed after only a few days of dosing the animals. Despite this limitation, there were still ample findings. Findings included decreased water consumption and dose-dependent decreased weight of exposed dams. There were also dose-dependent decreased litter sizes and decreased pup weights among exposed groups. Organ weights of livers, kidneys, adrenals, testes, and heart were decreased in the exposed groups and this disparity continued through PND 70. There were alterations to blood composition including dose-dependent increases in red blood cells, hemoglobin, hematocrit concentration, mean corpuscular volume, mean corpuscular hemoglobin and reticulocytes at PND21. Additionally, there was a dose dependent decrease in platelet volume, white blood cell counts, lymphocytes, large unstained cells, neutrophils, eosinophils, and cellularity of bone marrow observed from at PND21 and PND 70. The spleen and thymus weights of exposed animals in all exposure groups decreased and there were also effects
on splenocyte subpopulations including a decrease in natural killer (NK) cells, indicating potential immunotoxicity in the exposed animals.

3.5.1.5 Furan (Rehman et al., 2020)

Furan ($C_4H_4O$) is a chemical used by numerous industries; however, it is also found in day-to-day life including in food products that go through a heat treatment process such as canned/jarred foods, beverages, and infant formula. Additionally, furan can be found in cigarette smoke, wood smoke, or engine exhaust. The EOGRT study on furan is very limited. This investigation was conducted in rats that were dosed with furan orally at 0, 1, 2.5, 5, and 10 mg/kg. Males were dosed daily for 10 weeks before mating and females were dosed for 2 weeks before mating. Upon evaluation, researchers only investigated the reproductive parameters outlined in the test guidelines and only collected reproductive organs and clinical observations. The two highest treatment groups (5 and 10 mg/kg) were associated with decreased maternal body weights during prebreeding and gestation but increased during lactation. At the highest dose, there was a significant decrease in number of live births. At PND70, decreased testosterone was observed in F1 males and decreased estrogen levels were observed in F1 females. Additional reproductive findings were noted in the 10 mg/kg group including decreased ovarian and testicular weights, decreased sperm production and disturbed estrous cyclicity.

Researchers concluded that there is substantial evidence that furan exposure disrupts both parental and offspring hormonal function and regulation.

3.5.1.6 Polyhexamethylene (PHMG-P) (Lee et al., 2022)

PHMG-P is a polymer that is often used as a biocide in household products. In Korea especially, PHMG-P is used as a humidifier disinfectant biocide. The use of the
biocide in this way leads to increased and unintentional inhalation of the chemical (Lee et al., 2022). A large increase in pulmonary disease in Korea (Lee et al., 2022) led to the further investigation of this potential environmental toxicant. The EOGRT study on PHMG-P was fairly comprehensive although serum evaluations were omitted from the study. Pregnant females were exposed to PHMG-P for 6 hours/day via a whole-body inhalation chamber at target concentrations of 0, 0.14, 1.6 and 3.2 mg/m$^3$. These concentrations were based on epidemiological studies of human exposure to PHMG-P and were designed to account for physiological differences between rats and humans such as inhalation and respiratory rates. Although researchers mentioned a full evaluation of hematological parameters, none of these endpoints’ results were published. Dams exposed to the highest dose of PHMG-P had severe systemic toxicity as well as increased gestational periods. Additionally, offspring in the high exposure group were found to have a decreased viability index indicated by increased death rates. Decreased viability index and some systemic toxicities were also found at the 1.6 mg/m$^3$ exposed group. There was persistent low bodyweight observed in the exposed F1 generation, possibly correlated to decreased food consumption observed among the highest dose group. However, weight changes were the only significant finding reported in the F1 generation in this EOGRT study.

3.5.1.7 Nitrotriazolon (1,2,4-triazol-5-one; NTO) (Lent et al., 2016)

NTO is a chemical developed as a part of a new generation of insensitive munitions. The aim of these new compounds is to replace conventional explosive chemicals which are more susceptible to unintentional initiation. There has been increasing concern about insensitive munitions as they may contribute to adverse health
effects related to their release or production in the environment. Researchers investigating NTO followed the EOGRT guidelines but failed to include the neurotoxicity cohorts or immunotoxicity parameters. Rats were exposed to NTO via drinking water at 0, 144, 720, and 3600 mg/L and the F1 offspring the F1 offspring were directly dosed after weaning through puberty. This study focused on reproductive and developmental effects and all primary organs were weighed and then collected for histopathological analysis. Findings from this study pointed towards effects of NTO on reproductive development, especially in males. At the highest dose, F0 males and F1 male offspring both exhibited seminiferous tubule degeneration/atrophy. Additionally, the F1 males of this dose group had reduced weights of reproductive organs, increased nipple retention, and a 2.6-day delay in puberty.

3.5.1.8 Titanium dioxide (European Commission Report No. 36222, 2020)

The EOGRT study for titanium dioxide was conducted under the study guidelines by the European union’s ECHA. While the formal study is yet to be published, the preliminary findings have been published for public use. The study on titanium dioxide follows TG 443 with few deviations and the results were published in a methodical manner that reflected the guidelines. There were not any major findings in the titanium dioxide evaluation. Researchers noted slight changes at the highest dosage in estrogen levels of exposed F0 males and an increase of T3 levels in F0 females. The only findings in the offspring were a temporary and spontaneous change in food consumption in the low and medium doses. Further analysis is needed to determine the significance of these findings.
3.5.1.9 Vinclozolin (Schneider, 2011)

Vinclozolin is a dicarboximide fungicide used to control molds on produce including in vineyards. This chemical is a known anti-androgenic chemical and rats were dosed pre-mating through weaning via the diet at 0, 4, 20, and 100 mg/kg/day. Vinclozolin was evaluated under the EOGRT framework although there were some deviations from the guideline including failure to collect certain dam parameters such as water consumption, estrous cycle, and maternal/nursing behaviors. Additionally, researchers did not evaluate the erythrocyte count or total differential leukocyte in hematological analysis. Although researchers collected almost all secondary organs, they did not collect the mammary gland. Researchers found reduced weights of male reproductive organs in F0 and F1 males including alterations to the prostate, seminal vesicles, and epididymites at the 100 mg/kg/day dose. They additionally observed increased adrenal weights (high dose), reduced anogenital distance (high dose), and increased nipple retention (mid and high dose) in the F1 male groups. Histopathology of this group yielded evidence of hypospadias, purulent prostatitis, and inflammation with atrophy of the seminal vesicles. Lastly, exposed F1 females demonstrated accelerated vaginal opening.

3.5.2 Summary of unpublished studies using TG 443: Methimazole and Lead Acetate

Two studies were conducted and cited as TG 443 studies, but the published results are not available. Considering this important limitation, we have included the summary of the findings from these two studies because they were summarized in depth as a part of the article “Feasibility of the extended one-generation reproductive toxicity study (OECD
TG 443)” by Fegert et al (2012). Importantly, both of these studies have been used as part of a validation effort to demonstrate the strengths and limitations of the EOGRT TG.

Methimazole is a pharmaceutical that is widely prescribed to domesticated animals to treat hyperthyroidism, and it therefore is well-characterized for its properties of selective inhibition of thyroid hormone production. Rats were dosed via diet at 0, 15, 30, and 45 ppm which translates to intake of approximately 0, 1, 2, and 3 mg/kg/day. The study of methimazole was mostly comprehensive and assessment was performed in both male and female F0 and F1 cohorts although researchers did not measure the toxicokinetic properties of methimazole, conduct an auditory startle test, or measure the immunotoxicity response. There were several findings reported following exposure to methimazole in the F0 generation. Parental body weights were reduced at the mid and high dose groups, whereas food and water consumption were reduced at the highest dose. The length of gestation increased at the highest dose while the litter size decreased. There was a dose dependent increase in TSH and a decrease in T4, as expected from the known pharmaceutical uses of this drug. Thyroid hormone functions were accompanied by observed enlarged thyroids in all treatment groups and a dose-dependent increase in thyroid weight. Further histopathology of the thyroid gland indicated hyperplasia and hypertrophy at all doses.

In the F1 generation, researchers noted fewer effects including a dose-related reduction in body weight, delayed preputial separation at mid and high doses, and delayed onset of estrous at the high dose. Similar to the parental generation, there was a dose-related decrease in T4 and an increase in TSH and T3 as well as a dose-related
increase in thyroid weight and histopathological findings of diffuse follicular cell
hyperplasia/hypertrophy.

The second unpublished EOGRT study evaluated lead acetate, a compound used
as a fixative in dyes including dyes used in cosmetics and hair coloring agents in addition
to many other historical uses. Animals were exposed to lead acetate via drinking water at
0, 100, 800, and 1700 ppm. In the parental generation, parameters included mortality,
clinical signs, body weight, food intake, estrous cyclicity, and sperm parameters but
numerous endpoints were omitted including measurements of thyroid hormones and other
clinical pathology such as urine and blood analysis. All measurements were performed
for the F1 generation with the exception of auditory startle tests and immunotoxicity
responses. Lead acetate increased female parental body weights in the high-dose group
during lactation only, but slightly reduced body weights of F1 males and females at mid
and high dosages. Additionally, there was an observed delay in preputial separation in F1
males from the mid and high dose groups and a dose dependent reduction in all sperm
parameters. Microcytic anemia was noted in mid-dose males and high-dose females.
Mid-dose females and all high dose groups demonstrated decreased kidney weights at
PND 21 and PND 70. Researchers also reported reduced weight of male reproductive
organs but note it is likely attributed to reduced overall body weight. Finally,
histopathology of the peripheral nerve indicated an increased incidence of fiber
degeneration at PND 70 in the high dose group.
Table 3.1 EOGRT endpoint descriptions and estimation of timeliness of evaluation

<table>
<thead>
<tr>
<th>Body weight and consumption</th>
<th>Clinical observation (I/E)</th>
<th>Brief explanation</th>
<th>Time prior to necropsy</th>
<th>Time during necropsy</th>
<th>Time after necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>Clinical observation can include skin, subcutaneous, subcutaneous, or in-life-hormone analysis</td>
<td>Animals are weighed every 7 days</td>
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<td>0</td>
</tr>
<tr>
<td>Food consumption</td>
<td>Food is weighed every 7 days</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total consumption</td>
<td>Total is measured every 7 days</td>
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<td>0</td>
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</table>

<table>
<thead>
<tr>
<th>Maternal and pregnancy</th>
<th>Observational cycle</th>
<th>Brief explanation</th>
<th>Time prior to necropsy</th>
<th>Time during necropsy</th>
<th>Time after necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational length</td>
<td>The number of days between mating of males and females and the presence of a sperm plug is determined</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Duration of pregnancy</td>
<td>The length of time between sperm plug and parturition is determined</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Number of offspring</td>
<td>The total number of offspring is determined</td>
<td>+</td>
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<td>0</td>
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<tr>
<td>Length of pregnancy</td>
<td>The length of time is determined</td>
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<tr>
<td>Sperm availability</td>
<td>The number of sperm is determined</td>
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</table>

<table>
<thead>
<tr>
<th>Organogenetic development</th>
<th>Brief explanation</th>
<th>Time prior to necropsy</th>
<th>Time during necropsy</th>
<th>Time after necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neural tube closure</td>
<td>Neural tube closure in the embryonic or fetal period is determined</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ectopic growth</td>
<td>Ectopic growth is determined</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Neural tube closure</td>
<td>Neural tube closure in the embryonic or fetal period is determined</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Organogenesis</td>
<td>Organogenesis is determined</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>Spinal cord is determined</td>
<td>-</td>
<td>0</td>
<td>0</td>
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</table>

<table>
<thead>
<tr>
<th>Organogenetic development</th>
<th>Brief explanation</th>
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<th>Time during necropsy</th>
<th>Time after necropsy</th>
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<td>Neural tube closure in the embryonic or fetal period is determined</td>
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<tr>
<td>Ectopic growth</td>
<td>Ectopic growth is determined</td>
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<td>Neural tube closure in the embryonic or fetal period is determined</td>
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<td>Organogenesis</td>
<td>Organogenesis is determined</td>
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<tr>
<td>Spinal cord</td>
<td>Spinal cord is determined</td>
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<tbody>
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<td>Neural tube closure</td>
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<tr>
<td>Ectopic growth</td>
<td>Ectopic growth is determined</td>
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<tr>
<td>Neural tube closure</td>
<td>Neural tube closure in the embryonic or fetal period is determined</td>
<td>-</td>
<td>0</td>
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<tr>
<td>Organogenesis</td>
<td>Organogenesis is determined</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>Spinal cord is determined</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
3.5.3 Summary of endpoints included in studies utilizing TG 443

The EOGRT study was developed beginning in 2013. The guidelines were accepted for use and published formally in 2018 and are still recommended for use by the OECD. Of the 9 peer-reviewed studies, 4 closely followed almost all elements outlined in the guidelines. Two studies closely followed most aspects of the test guideline but omitted important blood serum evaluation. In all studies, regular clinical observations were performed on the dams. Additionally, collection and weight of the testis from the F1 generation males was included in all 9 studies.

Of the studies that were examined, the most common in-life endpoints that were evaluated were body weight (11 of 11), anogenital distance (9 of 11), the presence of nipple or areolae in males (11 of 11) and preputial separation (11 of 11). All of these measurements may indicate reproductive, developmental, or hormonal effects of the exposure of interest. All but two studies (9 of 11) evaluated the weight of the epididymides, testes weights, and sperm parameter endpoints. EOGRT endpoints of the liver, kidneys, spleen, thymus, and adrenal glands were evaluated by all studies except the Furan study. Finally, developmental immunotoxicity was evaluated in only 6 of the EOGRT studies.
3.5.4 Analysis of significant effects reported in studies using TG 443

We evaluated the findings from the eleven studies that reported using TG443 to determine if there were outcomes that were routinely found to be affected by the toxicants that have been evaluated. Six of the eleven studies reported altered body weights in one or both sexes of the F1 generation. Two studies (vinclozolin and 2,4
dichlorophenoxyacetic acid) found effects on the timing of puberty as measured by vaginal opening or preputial separation. Furthermore, two studies (vinclozolin and NTO) reported significant effects on altered weight of the epididymites and weight of the testes. Despite lack of effects on sperm parameters (motility and morphology), three studies reported effects on the weight of the prostate (vinclozolin, NTO and 2,4 dichlorophenoxyacetic acid).

**Table 3.4. EOGRT endpoints and study findings**

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Benzene acid</th>
<th>DOTC</th>
<th>2,4-dichlorophenoxyacetic acid</th>
<th>4-ethylphenol</th>
<th>Fennel</th>
<th>PEMS-2</th>
<th>NTO</th>
<th>Plasma chemistry</th>
<th>Viscera</th>
<th>Muscles</th>
<th>Muscular</th>
<th>Neural system</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
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<tr>
<td>Food consumption</td>
<td>X</td>
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<td>Preputial separation</td>
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<td>Penile length</td>
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<td>Penile circumference</td>
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<tr>
<td>Maternal body weight</td>
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<td>Neonatal food consumption</td>
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<td>Neonatal length</td>
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3.6 Discussion

3.6.1 Evaluation of consistency of endpoints evaluated in studies using TG 443

After conducting a comprehensive literature search with assistance from a library specialist, there were only 9 published and accessible studies found to have followed and cited OECD test guideline 443. An additional two studies were summarized in a feasibility study (Fegert et al. 2012). Of these 11 studies, only one followed every part of the guideline without deviation for the endpoints that should be evaluated. The remainder of the studies omitted certain criteria either due to the availability of previous research or without giving an explanation.

The purpose of OECD test guidelines is to have a standardized method of analysis to allow for comparability between studies and to ensure that data produced according to the guidelines can be utilized by regulatory agencies around the globe. The EOGRT guidelines intentionally leave room for deviation based on the chemical of study (e.g., doses and route of exposure) (Fegert et al., 2012). Deviations from the approved test guideline may make it difficult to compare results from EOGRT studies on different chemicals. Additionally, although some studies report that the study methods were conducted as per regulatory standards, in fact, the full complement of endpoints may not have been included. For example, there was a lack of serum evaluation and hematological parameters in the EOGRT study of Furan.

The OECD aimed to develop a guideline that could be used for safety/hazard evaluations across industries whereas before there were specific approaches used for pharmaceuticals, agrochemicals, and other toxicants. Avoiding redundant testing is
important for ethical reasons, e.g., to avoid the use of excess animals (Spielmann, 2009). However, redundant testing provides an opportunity to evaluate the reproducibility of findings in guideline studies, considering many of these tests are never repeated (Vandenberg et al., 2019, Vandenberg et al., 2020).

The EOGRT study design is complex and requires the right resources to conduct the study properly. Reviewers of the protocol recommended communication among labs registering EOGRT studies and regulatory agencies to ensure all needs or components are met, and the data are reported in a format that is most useful to regulators (Spielmann, 2011). The complexity of the EOGRT design also requires a substantial amount of existing data to determine elements of the study design such as selection of relevant dosages and route of exposure. This potential limitation is illustrated in the ethanol study. The doses that were selected caused severe systemic toxicity in the F0 generation to a point where researchers had to halt dosing. Determination of the ethanol dosages in this study may have been mis-calculated or drawn from incorrect data as highly lethal dosages are not practical for the EOGRT study design. Toxicity does not solely equate to lethality, leaving merit in evaluating the effects of sub-lethal dosages on such concerning chemicals.

3.6.2 Summary of significant effects observed in studies using TG 443

Although a large number of outcomes were evaluated across the eleven studies examined, alterations to body weight in F1 pups was the most common significant effect reported (6 of 11 studies), followed by two studies reporting effects on timing of puberty and two studies reporting effects on weight of male reproductive organs. Interestingly, even though several of the chemicals evaluated are described as EDCs, there were no
significant effects reported on sperm parameters. Importantly, other endocrine-mediated outcomes were not affected in any of the studies that utilized TG 443. For example, no studies reported alterations in ovarian weight. No chemicals altered the weight of the pituitary, reported changes in maternal or nursing behaviors, or altered the sex ratios of pups. The failure to detect effects of chemicals on these outcomes might mean that many (or most) of the chemicals that have been evaluated with TG 443 do not alter hormone action in the pathways implicated in these reproductive outcomes, or it might mean that these endpoints are insensitive measure of endocrine disruption. Future work is needed to determine which of these eleven chemicals have been studied in depth and if their mechanism of action has been identified.

Other endpoints incorporated in TG 443 that represent effects that may be mediated by the endocrine system include weights of the liver, kidney, spleen, brain, heart and thymus; weights of these organs can also be altered via non-endocrine mediated outcomes including systemic or organ-specific toxicity. Notable findings were mentioned in the DOTC and ethanol studies where researchers found alterations to the weight and histopathology of the spleen and thymus. Also, two studies (ethanol and 2,4 dichlorophenoxyacetic acid) reported alterations to the weight of the kidney. In contrast, no studies report alterations to the weight of the liver, heart or brain.

TG 443 allows additional “arms” of the study to be added to evaluate the potential for a chemical to have developmental neurotoxicity or developmental immunotoxicity. As described above, several of the published studies included these additional study “arms”. Although significant findings were reported in the DOTC and ethanol studies for immunotoxicity, no study observed significant effects on the neurotoxicity outcomes that
were evaluated. These results might suggest that the neurotoxicity outcomes included in TG 443 are not sufficiently robust or comprehensive, or alternatively, the chemicals that have been evaluated lack neurotoxicity effects. The guideline also describes a long list of secondary organs that can be collected from the F1 offspring in adulthood including peripheral nerves, muscle, spinal cord, optic nerve, gastrointestinal tract, mammary gland, bladder, lung, trachea, bone marrow, vas deferens, and the vagina. Again, several of the published studies included collections of these secondary organs but no study reported significant effects on these organs.

In the published EOGRT studies, researchers were able to report if observations persisted throughout life by conducting necropsies and collecting tissues at different time periods; in some cases, adverse outcomes were only observed during a certain life stage. For example, in the study of the humidifier disinfectant PHMG-P, researchers could confidently conclude that there was a persistent decrease in body weight in the F1 animals because these effects persisted across multiple age groups. In contrast, in the study of titanium dioxide, researchers noted changes in food consumption in the F1 generation, but upon follow-up concluded that this change was more likely spontaneous than related to exposure because it was not continuously observed.
3.6.2.1 How many published EOGRT studies included any measure of mammary gland toxicity?

Of the 9 reviewed EOGRT studies, 3 reported the collection or evaluation of the mammary gland: 2,4 dichlorophenoxyacetic acid, the humidifier disinfectant biocide PHMG-P, and titanium dioxide. However, in the PHMG-P study, it was reported if both male and female glands were collected, the method of microscopic analysis, or the results of their analysis (Lee et al., 2022). The study on titanium dioxide is not yet published, however preliminary results from the study were reported in a registered dossier from the European Chemicals Agency (ECHA). In this study, weights of mammary glands in female and male animals were reported and histopathological analysis was performed on the organ, however exact methods of analysis were not detailed in the dossier. The only reported finding from mammary gland analysis was the observation of a solid mass in one sample although the report did not elaborate on its diagnosis, relevance, or implications for interpreting the remainder of the mammary gland results (ECHA, 2020). Interestingly, the EOGRT validation study of 2,4 dichlorophenoxyacetic acid references
the evaluation of hormone levels and “endocrine sensitive glands,” however no direct mention of the mammary gland is provided.

3.6.3 Mammary gland evaluation in OECD guiding document 151

Guidance document 151 contains guidance on various elements of the EOGRT study. A summary of the guidance on methods for mammary gland evaluation is included in this thesis to later compare them to existing methods used in expert laboratories. The document describes the normal structure and function of the rat mammary gland and details the structures within the rat mammary gland that may indicate alterations following chemical exposures, noting that this organ is a sensitive indicator of endocrine disruption. For example, images of mammary glands affected by the known EDC 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) are included to juxtapose normal mammary tissue images. Additionally, the document details the mechanisms by which hormonal pathways influence the structure and function of the mammary gland and concludes with the recommended observations and grading criteria for the histopathological findings of mammary gland analysis. The document also suggests that many EDCs may not be directly carcinogenic to the mammary gland, but rather increase its susceptibility to carcinogenic insults. This same finding has been previously observed by the Russo group in their investigation of the effects of BBP on the rat mammary gland (see Chapter 2).

The guidance recommends that following euthanasia, researchers collect the fourth and fifth inguinal mammary glands and surrounding fat pads (including the inguinal lymph node in the sample). Samples should be whole mounted and evaluated for morphological changes. According to the OECD, in the male (rat) mammary gland, researchers should look for effects consistent with feminization or atrophy, marked by the
development of more female like structures or a reduction/increase in size and complexity of the mammary gland compared to the control group. In analysis of female samples, researchers should look for masculinization, atrophy, alveolar hyperplasia (the presence of hyperplastic epithelial structures), increased secretory material (increased fluid in alveolar regions or ducts), and ductal ectasia (changes in duct dilation). Finally, the OECD recommends a qualitative severity grading scale to denote changes in the mammary gland ranging from minimal to severe. The guidance document also provides recommended procedures for the histopathological analysis of the mammary gland including a recommendation that the gland be sectioned parallel to the skin, as opposed to the more common method of transverse sections. The drafted guidance encourages researchers to pay special attention to the development and persistence of terminal end buds as they serve as the precursor for mammary gland growth and development.

3.6.3.1 How do current EOGRT endpoints for mammary gland evaluation compare to endpoints evaluated in expert laboratories?

The OECD guidance on mammary gland collection and analysis is vague for evaluation of this complex and sensitive organ. They recommend a qualitative scaling of whole mount mammary glands to be graded on a scale from minimal to severe alteration when compared to control samples. In histopathological analysis the OECD recommends attention to alveolar hyperplasia, increased/decreased secretory material, ductal ectasia, and TEBs. In contrast, expert labs have developed quantitative and semi-quantitative measures to better classify changes in the mammary gland in both whole mounted and sectioned samples. In whole mounts for example, terminal end buds (TEBs) can be measured using imaging software to determine not only their number their as well
Researchers can also evaluate the area of the entire epithelial compartment, a measure referred to as the ductal area which allows researchers to compare the relative size of glands as an indication of increased or decreased development (Markey et al., 2001, Vandenberg et al., 2006). The complexity of the mammary gland can be quantified using similar measurement software to count the number of branching points in male or under-developed female glands or utilize methods such as the Sholl method, which uses software to calculate the complexity of the mammary gland via quantification of the number of branching points in concentric rings aligned outward from the nipple (Vandenberg et al., 2006, Stanko et al., 2015). The female adult mammary gland can become quite complex, and thus unbiased stereology methods can be used to quantify different structures within the gland. To conduct these analyses, images are taken of each sample at a consistent magnification between 20-40x. The image should be taken just anterior to the lymph node in the fourth inguinal gland. A 12 by 15-point grid is then overlaid onto the image and the researcher can identify each structure that lies at each crosshair: ducts, terminal ducts, alveolar buds, or different stromal components (blood vessels, adipose, periductal stroma). Then, using basic statistics, researchers can calculate the volume fraction comprised of different structures in the mammary gland.

Analysis of sectioned mammary gland samples (cut longitudinally, e.g., parallel to the skin) can provide further information to the researchers on effects of a toxicant on the mammary gland. Pathologists can measure the number, size and thickness of ducts; the size and density of adipocytes; and the thickness and composition of the periductal stroma surrounding the mammary ducts. Additional techniques can be used on tissue
sections to highlight endpoints related to mammary gland health. For example, toluidine blue stain reveals mast cells, an immune cell that is responsible for localized inflammation. Using immunohistochemistry, researchers can evaluate the percentage of cells expressing a protein or receptor of interest such as hormone receptors ERα and PR. This wide range of analytical methods can be conducted to gain a broader perspective of effects on the mammary gland as opposed to the visual qualitative analysis proposed by the OECD.

While expert laboratory methods of mammary gland analysis are more comprehensive, due to the limited reporting of mammary gland parameters in published EOGRT studies, a conclusion cannot yet be determined on which measures are more sensitive. Further research is needed to compare the sensitivity of each of these analysis methods to that of the OECD to determine if methods in current OECD practice is sufficient.

3.6.3.1.1 Comparing doses of observed mammary gland findings to other reproductive and developmental parameters

The first step in evaluating the sensitivity of the mammary gland as a measure for developmental, reproductive, and endocrine alteration is to compare effects of known EDCs that have been studied on the mammary gland to findings in studies that include similar outcomes to those in the EOGRT test guidelines. PFOA, BPA, and TCDD are three well researched chemicals that have been demonstrated to induce morphological changes in the mammary gland (White et al., 2007, Vandenberg et al., 2007, Fenton et al., 2022). These chemicals additionally have been shown to have other effects on reproduction and development, and although they have not been evaluated with the
EOGRT test guideline, they have been evaluated for outcomes that are similar to those endpoints that are found in the EOGRT study (Dixon et al., 2021, Tucker et al., 2015, Tassinari et al., 2019, Tyl et al., 2008, Cummings & Birnbaum, 1999, Bruner-Tran & Osteen, 2001).

In this preliminary investigation we first selected relevant and comparable studies on the three chemicals of interest. When comparing the doses of BPA and TCDD at which effects are observed, effects on the mammary gland were observed at doses several orders of magnitude lower than the doses that impacted reproductive/developmental parameters consistent with those included in the EGORT design (Figure 3.2). Such parameters include reproductive organ weights, histopathology, and function (OECD TG 443).

For PFOA, a different pattern has recently emerged. Numerous studies found effects of PFOA at a range of low doses. However, a recent study on PFOA found altered uterine weight after even lower doses (Dixon et al., 2021), below the doses that have been shown to alter mammary gland morphology and function (Tucker et al., 2015).

Our examination of these three chemicals was a preliminary approach to determine if the mammary gland might be more sensitive than other outcomes, including the endpoints that are included in standard test guidelines including the EOGRT. However, there are some important limitations to these findings. First, it is challenging to make definitive comparisons between the studies that evaluated mammary gland morphology and the studies that evaluated the other outcomes, because these results came from different studies conducted by different research teams, using different animals. While effort was made to only compare similar study designs, not all studies utilize the
same rodent model, timing of compound administration, and study protocol. Despite these limitations, comparing the findings of these studies demonstrates that changes to the mammary gland can be observed at extremely low doses of both BPA and TCDD. These doses are lower than doses reported to produce other reproductive-related effects in existing generational studies (York et al., 2010, Wang et al., 2020).

To further assess and compare the sensitivity of the mammary gland compared to other endocrine and reproductive end points, comprehensive studies must be conducted that include traditional methods of evaluating toxicity and mammary gland morphological analyses. This current analysis however provides the justification for such studies considering the low doses at which effects have been observed in the mammary gland in this preliminary investigation.
Comparing doses with observed effects across studies of known EDCs PFOA, BPA, and TCDD. Endocrine related affects were observed at PFOA doses of .01, .3, 1, and 3 mg/kg bw. Affects were observed at BPA doses of .00025, 6, 18, and 600 mg/kg bw. Affects were observed at TCDD doses of .0001, .0004, .001, .003 mg/kg bw.
Table 3.6 Authors, animal models, and included doses in studies utilized in figure

3.2

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<th>Chemical</th>
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<th>Model</th>
<th>Doses studied</th>
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<tr>
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<td>CD1 mice</td>
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<td>5 mg/kg bw/day</td>
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<td>.003, .03, .3, 5, 50, 600 mg/kg bw/day</td>
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<td>TCDD</td>
<td>Cummings &amp; Birnbaum 1999</td>
<td>Sprague-Dawley rats and C57BL/6 female and C3H males</td>
<td>1 (rats) or 3 (mice) μg/kg. 0, 3, or 10 μg/kg at 3, 6, and 9 weeks (both)</td>
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<td>TCDD</td>
<td>Bruner-Tran &amp; Osteen 2011</td>
<td>C57BL/6 mice</td>
<td>10 μg/kg (in utero), 10 μg/kg (in utero &amp; 4 weeks), 10 μg/kg (in utero, 4 weeks, &amp; 9 weeks)</td>
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3.6.3.2 What is the evidence that the altered developmental parameters and/or malformations detected in the whole mount mammary glands are toxicologically adverse effects?

In terms of the mammary gland, toxicologically adverse outcomes can be defined as outcomes that induce morphological changes that can affect functionality or increase susceptibility to carcinogenesis (Osborne et al., 2014). Morphological changes can be observed in whole mount analysis such as overall growth and complexity of the mammary gland as well as changes to the normal proportions of epithelial structures; because the mammary epithelium is where most breast tumors arise, an increase in the number of epithelial structures has been proposed to be a risk factor for mammary cancer. As mentioned in the EOGRT guidance document, TEBs are of interest to researchers as
these are highly proliferative structures, making them sensitive to carcinogenic compounds. Observation of increased TEBs is an indication of increased susceptibility to carcinogenesis, fitting with the description of an adverse outcome (Fenton 2009). In contrast, reduced mammary gland growth or complexity may have functional implications during lactation. The lack of epithelial structures may contribute to reduced milk production or altered milk composition. Prior studies from the Vandenberg lab and others suggest exposures to some EDCs during either the pregnancy/lactational period or during gestation can alter the differentiation and full development of the lactational mammary gland, leading to under-sized pups and/or necessary changes in maternal behavior to provide pups with sufficient nutrients.

Another key example of toxicologically adverse effects is seen in a study of BPS, a known ER agonist. LaPlante et al. evaluated its effects on the lactating mammary gland’s morphology and on nursing behaviors. BPS reduced the volume fraction of lobules, the structures responsible for milk production, in mice exposed during pregnancy and lactation (LaPlante et al., 2017). Additionally, there were effects on both maternal and pup nursing behaviors. BPS exposed pups were less likely to initiate nursing and BPS exposed dams (200 μg BPS/kg/day) spent more time nursing during the later lactational period consistent with a behavioral adaptation to a lactational defect. These findings provide evidence that BPS exposure may induce early involution in the mammary gland and have potential consequences for offspring due to altered maternal behaviors.

A final example of a mammary gland findings that should be considered adverse is PFOA. Research on PFOA exposure in pregnant mice found that mice exposed during
pregnancy had mammary glands that were less differentiated compared to control mice. Upon further investigation, these F0 females demonstrated delayed involution and altered milk protein gene expression (White et al., 2007). Effects of PFOA exposure on the pregnant mammary gland further extended into the offspring (F1 generation), which had stunted branching and growth of the mammary gland among exposed animals compared to control (White et al., 2007). Another study evaluated the effects of developmental BPA and DES exposure on mammary gland differentiation and function (Kass et al., 2012). Gestationally exposed Wistar rats were raised into adulthood, bred, and evaluated for their pregnancy and lactation outcomes. In terms of mammary gland structure, there was a delay in histological differentiation of the mammary gland in the treated animals accompanied by altered milk yield patterns (Kass et al., 2012). Furthermore, F1 female rats had altered milk protein composition during lactation (long after their initial exposure) (Kass et al., 2012). These studies indicate that changes to the morphology of the mammary gland may produce adverse effects if they affect the functionality of the gland.

3.7 Conclusions

In the scope of this thesis, we answered preliminary questions that must be addressed before proceeding with assessments of the sensitivity and reproducibility of the current EOGRT MG analysis methods compared to those used in expert labs. The first question of interest was which of the published EOGRT studies included any measurement of mammary gland toxicity. In our investigation we found that 3 studies reported collection and evaluation of the mammary gland. However, none of these studies reported findings or detail on methods of analysis.
The second question answered in this thesis is how the current EOGRT and OECD methods of mammary gland analysis compare to those used in expert laboratories. Methods of evaluation set forth by the OECD are largely qualitative. Additionally, the guiding documents detailing the analysis methods are vague and may be difficult to conceptualize without existing knowledge. Methods utilized in expert laboratories include quantitative, semi-quantitative, and qualitative assessments. These established methods have clear protocols and additionally require basic equipment that most animal analysis laboratories would have access to.

The final question addressed by this thesis is regarding the evidence that altered developmental parameters of malformations detected in the whole mounts are toxicologically adverse. In answering this question, we exemplify how alterations to mammary gland development and function fit into the definition of adverse effects. Additionally, we provided specific examples of experiments which found morphological mammary gland changes and the associated adverse effects to lactation or maternal behavior.

There are still questions that remain unanswered but may be pivotal when weighing the arguments for implementing the mammary gland (and updated evaluation methods) as a primary endpoint in the EOGRT study. One remaining question is if the mammary gland endpoints that have been evaluated in expert labs are more sensitive measures of toxicity than the mammary gland endpoints that are already included in the TG. Answering this question will require a quantitative comparison of doses that alter mammary gland morphology with the doses that alter mammary gland outcomes utilized in the EOGRT study including mammary gland weight and standard measures of
histopathology. Additionally, we must evaluate if the mammary gland and its analysis methods are more sensitive than other endocrine endpoints that are currently included in the TG. Answering this question will require a quantitative comparison of the doses that affect mammary gland outcomes with the doses that affect other measures of toxicity, including measures of endocrine disruption such as altered reproductive organ weights.

Finally, we must determine how feasible it would be to add improved measures of mammary gland health/disease to TG 443. Perhaps there may be other outcomes included in the current guidelines that could be removed for the sake of adding better mammary gland analysis methods. One of the challenges to adding better measures of mammary gland health/disease to the TG is a concern that the mammary gland outcomes are not feasible for non-experts, or are too time-consuming, or are too expensive. To preliminarily address these concerns, we estimated the time commitment for conducting the endpoints that are already included in TG 443. In our preliminary estimation, the mammary gland would not create feasibility concerns, but future evaluations will be needed to produce a more quantitative assessment of the time and effort needed by labs that are currently considered non-experts. The final component of feasibility is the development of training materials by mammary gland experts to ensure that non-experts can properly extract and evaluate the organ. The Vandenberg lab has begun to create such materials, in concert with experts from the National Toxicology Program and NIEHS, and other experts in the European Union.

Finally, there are scientific questions that need to be addressed before the mammary gland whole-mount analysis can be added to the TG. Most importantly we must address the reproducibility of MG toxicity measures within experimenters and
between experimenters. The Vandenberg Lab previously evaluated our whole mount mammary gland analysis method for its reproducibility and accuracy within and between researchers of varying experience levels. We found that following basic training in measuring whole-mount mammary gland parameters, novice researchers were able to reasonably reproduce the findings produced by an expert researcher (Vandenberg et al, 2020). To further verify the reproducibility of mammary gland whole mount analysis, methods must be compared between labs. This can be done using a round-robin method where a set of de-identified mammary gland images are sent to multiple laboratories for evaluation. Results will be evaluated within researchers and then further compared between labs to see if their findings are similar (indicating reproducibility of evaluation) or different (indicating methods are not reproducible). Such a quantitative evaluation will help to further determine if the mammary gland is a worthwhile addition to TG 443.


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